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List of abbreviations

General abbreviations	
% (v/v)	% (volume/volume)
% (w/v)	% (weight/volume)
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bp	base pair
CAA	Casamino acids
CAF	Ammonium iron citrate
c-di-AMP	Bis-(3'-5')-cyclic dimeric adenosine monophosphate
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleic triphosphate
et al.	Et alii
EV	empty vector
Fig.	Figure
LB	Luria-Bertani medium
LC-MS	Liquid-chromatography mass-spectrometry
LFH	Long flanking homology
MSSM	Modified Sodium Spizizen Minimal medium
NADH/NAD^+	Nicotinamide adenine dinucleotide
NADPH/$NADP^+$	Nicotinamide adenine dinucleotide phosphate
OD	optical density
ONPG	o-Nitrophenol-β-D-galactopyranosid
PCR	polymerase chain reaction
rpm	rounds per minute
RT	room temperature
SP	Sporulation medium
TCA	Tricarboxylic acid
WT	wild type
Δ*gene*	Deletion of the respective gene

Chapter 1 – Abstract

Amino acid metabolism is a a central field of research aiming to understand the complex reactions that take place in a cell. Amino acids are the main building blocks for proteins, and they serve as carbon or nitrogen source and are therefore ubiquitous throughout all life forms. All amino acids apart from glycine possess an L- and D-enantiomer. While proteins are exclusively synthesized from L-amino acids, D-amino acids still play a big role in bacteria and are an upcoming field of research. Acquisition of amino acids occurs either by synthesis or transport. The gram positive soil bacterium *Bacillus subtilis* was used as a model organism for decades in research, as it is able to synthesize all amino acids by itself and also to take them up from the environment. However, the transporters for each amino acid are still not known. In fact, no transporters for glycine, phenylalanine, tyrosine and asparagine have been identified so far. Additionally, several amino acids are toxic to *B. subtilis* under specific conditions or even already to the wild type. This work used amino acid toxicity to uncover the mechanisms employed by the cells to deal with L-histidine, L-asparagine and D-asparagine. For this purpose, the c-di-AMP free (Δ*dac*) strain was used in conjunction with amino acid stress in order to trigger the formation of suppressor mutants. When stressed with L-histidine, the cells responded with mutations in the *azlB* gene, encoding for the transcriptional repressor AzlB. The expression analysis revealed an overexpression of the bipartite amino acid exporter AzlCD, which was also verified as histidine exporter via liquid chromatography-mass spectrometry. When L-Asn stress was applied, the Δ*dac* acquired mutations in the potassium transporter KtrD, showing a possible link between asparagine and potassium metabolism. Furthermore, deleting *aimA* also provided resistance to L-Asn, establishing it as L-Asn importer. In order to further elucidate asparagine metabolism in *B. subtilis* the reactions to L-asparagine were examined in an asparaginase-deficient strain (Δ*ansAB* Δ*ansZ*). The experiments again revealed suppressor mutations in *azlB*. This suggests a role of AzlCD in L-asparagine export and cements its role as a broad range amino acid exporter. Further adaptation experiments revealed mutations in *aimA*, as well as *bcaP,* which both led to increased resistance to the L-asparagine stress. These two were already known to be broad range transporters in *B. subtilis*. The data proves the role of AimA and BcaP in L-asparagine uptake. Suppressor screens with D-asparagine revealed mutations in *mleN*, coding for the malate/lactate antiporter MleN. Subsequent growth experiments could verify that MleN is the main transporter for D-asparagine, as the deletion strain fully complements growth during D-asparagine stress. A role of MleN in L-asparagine transport was not found. This work therefore suggests that stereo-enantiomeric amino acids are not necessarily taken up by the same uptake system. This work takes a step forward in the understudied field of amino acid transport and discusses amino acid toxicity, while providing strategies to identify novel transporters in the future.

Chapter 2 – Introduction

2.1. Amino acid transport in *B. subtilis*

2.1.1. Basic principles of amino acid transport

Amino acids are the individual compounds of every functional enzyme and thus are a necessary component of every living organism. There are 22 proteinogenic amino acids, of which 20 are encoded by the universal genetic code, meaning they take part in protein biosynthesis. Some prokaryotes, like *B. subtilis* are able to synthesize each amino acid by themselves. This ability was lost during evolutionary processes, as higher organisms like humans cannot synthesize every amino acid anymore and therefore rely on nutrition to cover the essential amino acid demand. Apart from synthesis, bacteria also have the ability to take up amino acids from the environment. In order to enter the cell, the solutes have to pass the semipermeable bacterial membrane. This is made possible by various transport systems, carrier proteins and permeases, which are embedded into the membrane and have different functionalities. In a uniport process, a metabolite is able to pass through the membrane unidirectionally by itself. In *B. subtilis* an uniporter is responsible for calcium uptake into the cell, driven by the membrane potential (Kusaka and Matsushita, 1987; Matsushita et al., 1989). Symporters require the transport of two solutes in the same direction. This process therefore was labelled "cotransport". *B. subtilis* possesses numerous symporters, with one of the most important being AimA, the major low-affinity amino acid importer, which also takes up glutamate (Klewing et al., 2020; Krüger et al., 2020). Symporters often rely on positively charged cations like H^+ and Na^+ to enable the transport. The last transport system are antiporters. Here, one solute is transported into one direction, while another solute is transported in the opposite direction. The malate/lactate antiporter MleN is an example for this process, as the protein takes up malate, while simultaneously exporting lactate (Wei et al., 2000). Transport processes happen along a concentration gradient. If the extracellular concentration is higher than the intracellular concentration, the substrates are passively entering the cell via facilitated diffusion. In this case, carrier molecules help the passing of the cell membrane as they bind exterior substrates and then release them into the cytoplasm. This happens passively, however active transport processes are also carried out by the cell, when substrates are transported from the site of lower concentration to the site with higher concentration. These processes require energy, which is gained by ATP hydrolysis or by proton motive force. Active transport is achieved by numerous transporter proteins in *B. subtilis*, for example by ATP-binding cassette (ABC) transporters. To form a functional transporter, multiple subunits form a complex, which then enables the import. At one part of the complex, ATP is broken down to ADP, which generates the energy necessary to import the respective substrate. GlnHMPQ, the glutamine transporter is a good example for such a system

(Quentin et al., 1999). The general structure of amino acid transporters is conserved amongst bacteria with a division into several classes/families. The largest family of transporters in *B. subtilis* is the Amino Acid-Polyamine-Organocation (APC) superfamily, which includes 22 known members. AimA, the major low affinity amino acid importer also belongs to this group. Currently, 49 proteins are included in the group of amino acid transporter in *B. subtilis* (Pedreira et al., 2022), but not all of them are well researched and characterized.

2.1.2. Known and unknown transporters in *B. subtilis*

B. subtilis has been a well studied model organism for a few decades now. Still, the field of amino acid transport marks an understudied field of research, as several amino acids, namely L-asparagine, L-phenylalanine, L-tyrosine and L-glycine have no transporter assigned to them (Wicke et al., 2023). For L-lysine it is only hypothesized that it is taken up by YvsH, a transporter within the APC superfamily that is regulated by the L-Box riboswitch (Rodionov et al., 2003). The existence of amino acid transporters for every amino acid is guaranteed, as one can delete every amino acid synthesis gene that catalyzes the last reaction to form the respective amino acid. The result is a strain that is auxotrophic for the respective amino acid and therefore dependent on uptake systems. An overview of amino acids and their respective importers as well as exporters can be seen in table 1.

Table 1 Overview of known and unknown amino acid importers and exporters in *B. subtilis*. (Sidiq et al., 2021; Belitsky, 2015; Hullo et al., 2004; Sarsero et al., 2000; Burguière et al., 2004; Zaprasis et al., 2014; Moses et al., 2012; Klewing et al., 2020; Krüger et al., 2020; Zhao et al., 2018; Satomura et al., 2005)

Amino acid	Importer	Exporter
Non-polar amino acids		
L-Alanine	AlaP, AimA	?
L-Valine	BcaP, BraB	?
L-Leucine	BcaP, BraB	?
L-Isoleucine	BrnQ	?
L-Methionine	MetNPQ	?
L-Phenylalanine	?	?
L-Tyrosine	?	?
L-Tryptophan	TrpP	?
Cystine	TcyABC, TcyJKLMN, TcyP	?
Glycine	?	?
L-Proline	OpuE, PutP, GabP	?
Polar amino acids		
L-Serine	AimA, BcaP, YbxG	?
L-Threonine	BcaP, YbxG	?
L-Asparagine	?	?
L-Glutamine	GlnT, GlnQHMP, AlsT	?
Positively charged amino acids		
L-Arginine	ArtPQR, RocC, RocE	?
L-Histidine	HutM	AzlCD
L-Lysine	(YvsH)	?
Negatively charged amino acids		
L-Aspartate	GltT, YveA	?
L-Glutamate	AimA, GltT	?

This illustrates that there is still a lot of knowledge to be acquired in the field of unknown transporters. AzlCD is the only described exporter of proteinogenic amino acids in *B. subtilis* (Meißner et al., 2022), although it is likely that more amino acid exporters exist, as there are a few homologs to known exporters of other organisms. YisU, a protein homologous to the *Corynebcaterium glutamicum* LysE

export protein, as well as YrhP, a homolog to *Escherichia coli's* RhtB, might be involved in L-arginine and L-lysine, as well as L-threonine and L-serine export, respectively (Saier et al., 2001). The EamA-exporter family just recently became a research topic. It was shown, that there exist multiple broad range exporters in *B. subtilis*, which are tightly regulated by transcription factors (Warneke et al.,, personal communication). This principle is further discussed in the next part of the introduction. As an addition to amino acid import and export not being fully explored, there also are numerous uncharacterized proteins in *B. subtilis*, which share the common amino acid transporter structure, yet no transported substrate could be identified yet. These proteins are summarized in Figure 1.

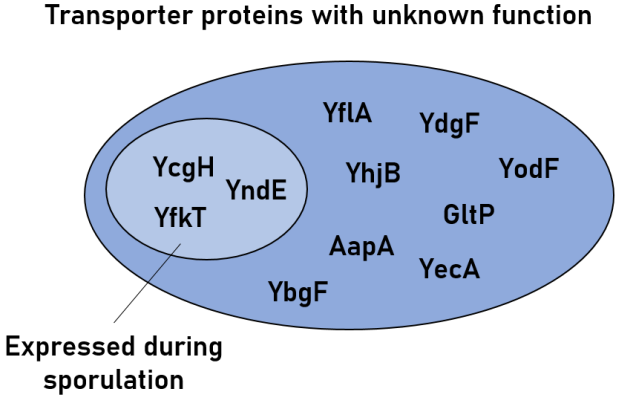

Figure 1 Taken from (Wicke et al., 2023) Overview of transporter proteins with unknown function. While some of these proteins are only expressed during sporulation, others are moderately expressed under standard growth conditions.

2.1.3. Expression of amino acid importers and exporters

The gene expression profile of all genes in *B. subtilis* was analyzed for multiple different conditions (Nicolas et al., 2012). This allows a general estimation, at what condition the respective protein is needed and expressed. While most essential genes are highly expressed under most conditions, this is not the case when it comes to amino acid transporters. A comparison of the essential *fusA* gene, which acts in translation and the *aimA* gene, which is the major low affinity amino acid transporter in *B. subtilis* illustrates the difference in expression (Fig. 2). The majority of amino acid transporters is poorly expressed under standard conditions, but can be induced, if excess substrates are present in the medium. This was shown for the glutamine uptake transporter GlnT, which is expressed in the presence of glutamine (Satomura et al., 2005).

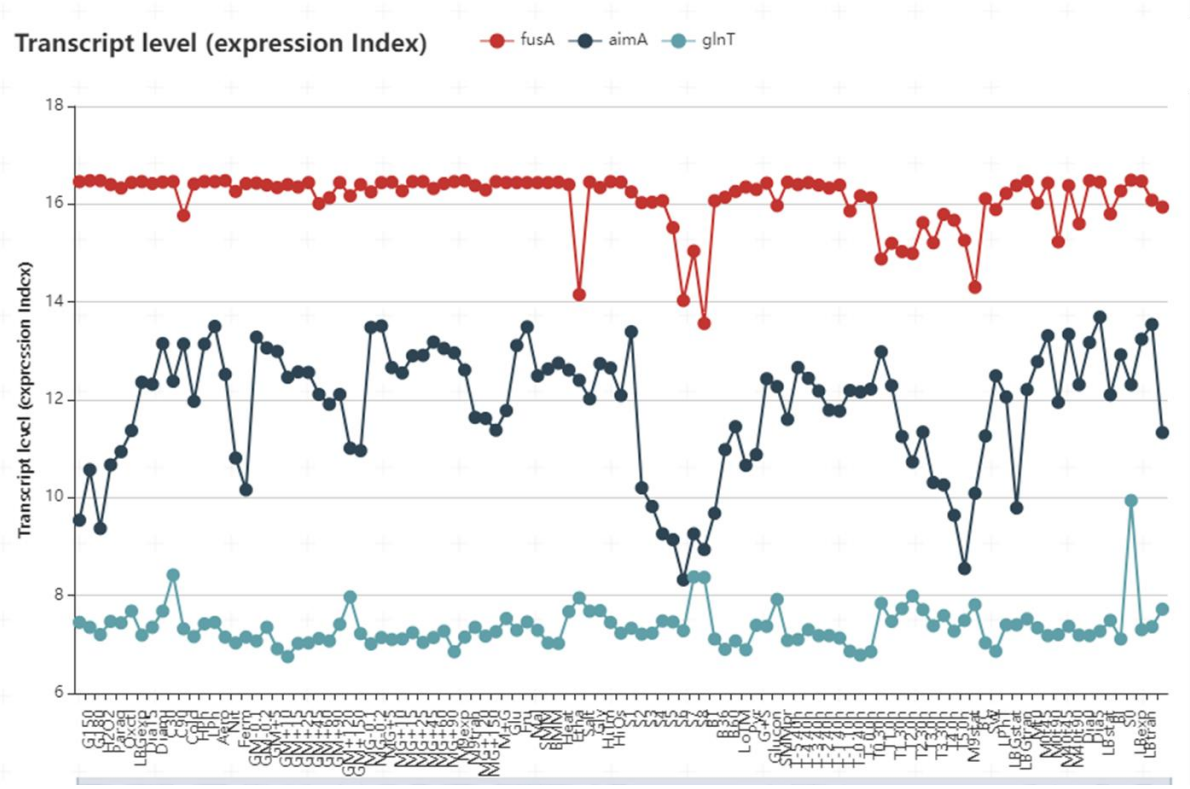

Figure 2 Transcription levels of the *fusA* (red), *aimA* (dark blue) and *glnT* (teal) genes over multiple tested conditions according to Nicolas et al., 2012. The essential *fusA* gene is highly expressed at all conditions, while the *aimA* gene, coding for the main amino acid transporter is only moderately expressed. Specific transporter genes like *glnT* are not expressed under standard conditions and are only induced upon availability of their ligand substrate. Figure generated with *Subti*Wiki.

This shows, that *B. subtilis* prefers glutamine uptake over self-synthesis, as GlnA, the glutamine synthetase is feedback inhibited (Schreier et al., 1989) and thus only active if not enough excess glutamine can be taken up from the environment. This solidifies the importance of unregulated amino acid import by broad range amino acid importers like AimA, which are constantly expressed and help sustaining the proper intracellular amino acid concentration. The counterpart to this is amino acid export, which is tightly regulated by an interplay of regulators and the actual export proteins. Recently, it was shown, amino acid export in *B. subtilis* is carried out via broad range exporters, like AzlCD, which exports branched chain amino acids, histidine, azaleucine and potentially more amino acids (Belitsky et al., 1997; Meißner et al., 2022). In this example, the export is only active, when the transcriptional repressor of the operon, AzlB, acquires mutations and is therefore rendered unfunctional. The subsequent overexpression of the operon then allows for enhanced export of amino acids. The regulation of exporters can also occur via activators and is conserved between species, as demonstrated by Lrp in *Corynebacterium glutamicum*, which activates the two component exporter BrnFE (Lange et al., 2012). This ensures amino acid export only under specific conditions, to avoid unnecessary loss of amino acids, as most exporters are also not limited to only one substrate. Another possibility of export regulation is demonstrated by BrmB in *B. subtilis*, which controls the expression

of the multidrug ABC transporter BmrCD, via a ribosome-mediated transcriptional attenuation mechanism, (Reilman et al., 2014) as it is co-expressed with BrmCD. Apart from this regulation mechanism, the export of BrmCD is also controlled by AbrB, the transition state regulator of *B. subtilis* (Olson et al., 2014). AbrB acts as a repressor, which adds a second regulation mechanism and thus further reinforces the importance of tight expression regulation, even of antibiotic exporters, like BmrCD. Just recently, an interesting family of exporters has gained a spike in interest. The proteins of the EamA-exporter family are all not expressed under standard conditions. Furthermore, it was found out that these exporter proteins are depending on mutations in the transcription factors lying directly upstream in a genetic context (Warneke, personal communication). This happening was described as "sleeping beauty" phenomenon as the respective exporters seem to export a broad range of amino acids very efficiently, but only if the mutation in the transcription factor occurred beforehand.

2.1.4. Challenges of amino acid transport

Although the general transporter structure is conserved amongst bacteria, the characterization and identification of novel amino acid transporters still encounters numerous challenges: (I) The substrate specificity of most transporters is low, causing them to take up amino acids and other metabolites at once, which might not share big structural uniformity. (II) One amino acid is taken up by a number of different transporters. (III) Lastly, the affinity of every transporter varies, resulting in some transporters only being active under specific conditions (Figure 3) (Wicke et al., 2023).

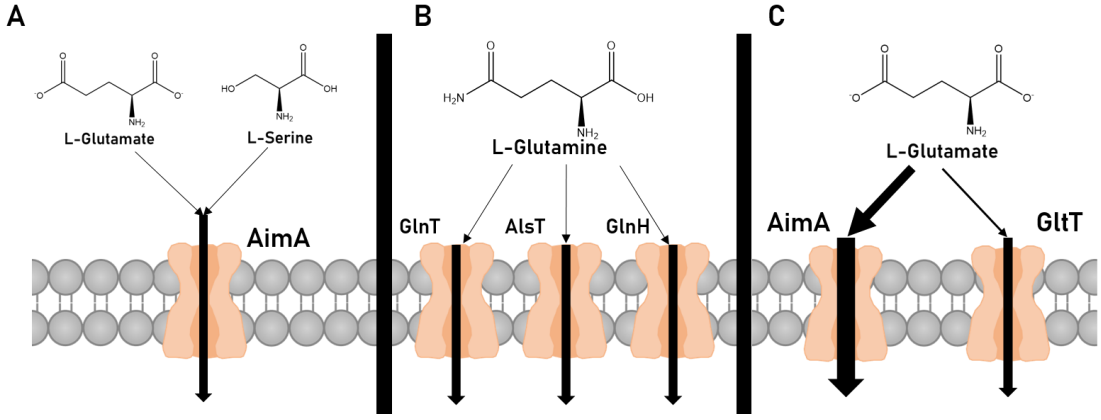

Figure 3 Challenges of amino acid uptake in *B. subtilis*. A Different amino acids enter the cell via the same transporter. AimA is the major importer for glutamate as well as serine. **B** One amino acid is taken up by multiple different transporters. **C** Different transporters possess different affinities towards the same amino acid.

2.2. The importance of glutamate for amino acid metabolism in *B. subtilis*

Among all amino acids, glutamate stands out for multiple reasons. The cytoplasm of a typical prokaryotic cell like *E. coli* or *B. subtilis* can be seen as a potassium-glutamate solution, as these two ions are present in very high abundance, reaching concentrations of up to 150 mM (Epstein, 2003;

Bennett et al., 2009; van Eunen et al., 2010; Meers et al., 1970). While potassium is needed, to buffer the general negative charge of the DNA, glutamate serves as amino group donor for nitrogenous compounds and thus provides a link between carbon and nitrogen metabolism. This link is illustrated by the fact that glutamate is needed to assimilate nitrogen (Merrick and Edwards, 1995). *B. subtilis* relies on the interplay of the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway to assimilate nitrogen into the metabolism. The glutamine synthetase (GS), encoded by *glnA* in *B. subtilis* utilizes glutamate and ammonia to form glutamine and the glutamate synthase (GOGAT), encoded by *gltAB* in *B. subtilis* transfers the amide group from glutamine to 2-oxoglutarate to produce glutamate. Apart from this pathway, there also exists the glutamate dehydrogenase (GDH) pathway to assimilate nitrogen. In its reaction, ammonia and 2-oxoglutarate are used to form glutamate. The intracellular pool of glutamate has to be kept high at all times to ensure its availability as a building block in biosyntheses. In *E. coli*, glutathione, a metabolite that is formed from glutamate is used to maintain the redox homeostasis of the cell and provides protection against oxidative damage (Prinz et al., 1997). Glutamate is also a good C-source for *B. subtilis*, as it is metabolized to 2-oxoglutarate, which directly feeds into the TCA. In the laboratory strain 168, the concentration of 2-oxoglutarate is low and maintained by RocG, as the other glutamate dehydrogenase GudB is cryptic in this strain and requires mutations to be decryptified (Gunka et al., 2012). GltAB, the glutamate synthase also plays a role in the balance of Glu/2-OG (Jayaraman et al., 2022). A Δ*gltAB* mutant becomes auxotrophic for glutamate, as the last step in glutamate synthesis is missing, but a lot of amino acid degradation pathways also lead to glutamate. Metabolization of glutamine, histidine, arginine, aspartate and asparagine, ornithine and proline directly lead to glutamate and alanine is thought to be linked to the glutamate pool as well (Sidiq et al., 2021). This marks glutamates role as a central point in amino acid metabolism.

2.3. Amino acid toxicity

2.3.1. General amino acid toxicity and toxic analogs of amino acids

Even though L-amino acids are necessary to build up a functional organism, accumulation of amino acids can be harmful to bacteria. L-Serine was found to be toxic to wild type cells of *B. subtilis* already at a concentration of 244 µM (Klewing et al., 2020). L-threonine also inhibits growth and sporulation of *B. subtilis* at concentrations of 4-8 mM, as it limits the synthesis of valine. This happens, as a degradation product of threonine, 2-oxobutyrate competes with pyruvate for the active site of the acetohydroxyacid synthase, which disturbs the natural flow of carbon to form valine (Lamb and Bott, 1979b; Belitsky, 2015). In general, it can be assumed that the reason for the toxicity lies in the high reactivity of amino acids, as they, or their degradation products can interfere with enzymatic reactions (de Lorenzo et al., 2015). Glutamate itself, even though being the most abundant amino acid can have

harmful effects on the cells (Commichau et al., 2008). The effect of glutamate toxicity is elevated in a Δ*rocG* mutant, since the high concentrations of glutamate cannot be efficiently degraded without a functional glutamate dehydrogenase (Gunka and Commichau, 2012). This intracellular accumulation of amino acids might also disturb the osmotic balance of the cell (Clark, 1985). However, the exact reasons for amino acid toxicity often cannot be narrowed down, as amino acids have global rather than pinpoint effects in the bacterial cell. The structure of amino acids is quite simple, causing them to often possess analogs with a similar structure. These analogs often are toxic to the cell, as they are mistakenly entering metabolic processes, rendering them unfunctional. This finding was already described in 1967 (Fowden et al., 1967), listing numerous toxic analogons to proteinogenic amino acids, while describing their mode of action. A common event is the misincorporation of the analogous amino acid into the polypeptide chain during protein biosynthesis, which is lethal to the cell (Rodgers and Shiozawa, 2008). Due to their structural similarity, analogs of amino acids can enter the cell via the same transporter as the actual amino acid, as was reported in multiple cases (Klewing et al., 2020; Gollnick et al., 2001; Zhu et al., 2009). This can be used to trigger suppressor mutations, which affect the respective importer of the amino acid.

2.3.2. D-amino acids

All proteinogenic amino acids with the exception of glycine have a chiral center, a central carbon atom that can be seen as a mirror axis. The L- and D variant of an amino acid carries the same functional group, but it is orientated in the mirrored direction, creating enantiomers with the prefix L- and D- (Figure 4).

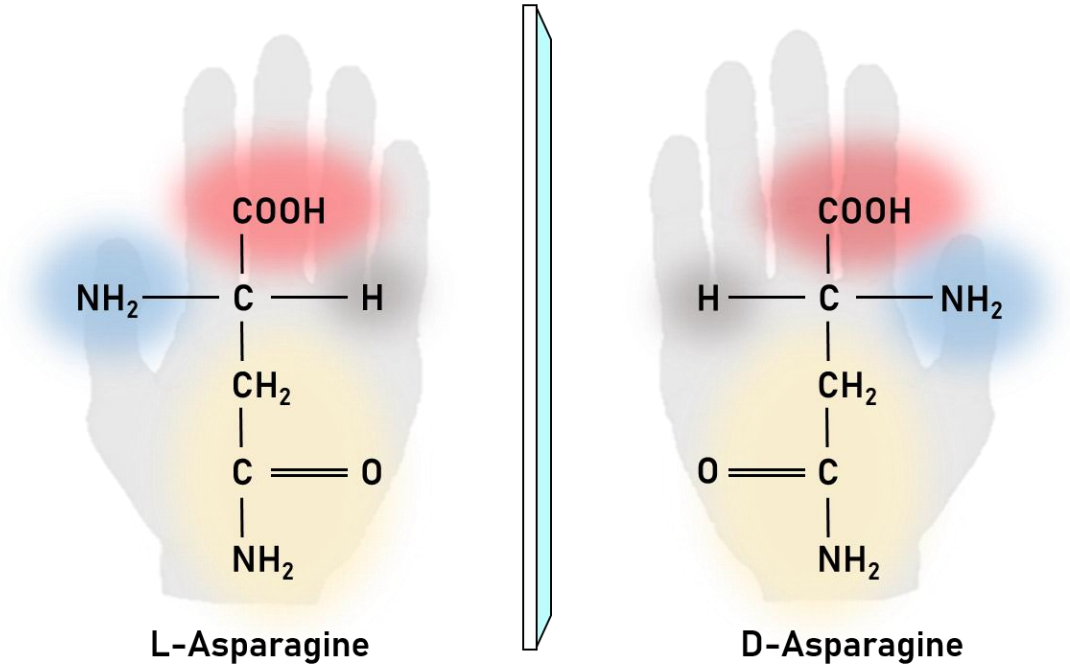

Figure 4 Chirality of amino acids. The central C-Atom has a covalently bound Carboxy- and Hydroxygroup, as well as a Hydrogen atom and the rest group. L-asparagine is a proteinogenic amino acid, while D-asparagine is toxic to *Bacillus subtilis*.

The ribosomes during protein biosynthesis exclusively use the activated tRNAs, which carry L-enantiomers of amino acids to form a polypeptide chain. This implies that all functional enzymes of every living organism are naturally composed of L-amino acids. Advances in synthetic biology have made it possible to integrate unnatural amino acids at specific sites of a protein (Noren et al., 1989), but peptides containing D-amino acids are the result of either posttranslational modifications (Soyez et al., 2000) or synthetic synthesis processes, which do not involve a ribosome (Luo et al., 2002). The modification of ribosomes to enable a sensible integration of D-amino acids into proteins is still subject of current research (Dedkova et al., 2006; Lander et al., 2023). Thus, even though the possible existence of a mirror-image life was postulated already in 1905 (Louis Pasteur, 1905), it still is a late-breaking topic of modern synthetic biology. Nowadays, D-peptides have entered a vast array of research fields, as they are discussed to be used as therapeutics in cancer therapy and HIV-treatment (Lander et al., 2023; Welch et al., 2007). Even though the ratio between L- and D-amino acids is heavily shifted towards L-amino acids when it comes to general abundance (Brack et al., 2008), D-amino acids are also part of the natural environment and biosphere. The cell wall of *B. subtilis* contains D-glutamate, D-aspartate as well as D-alanine, which allows the formation of crosslinks between multiple molecules of peptidoglycan (Angeles and Scheffers, 2021; Vollmer et al., 2008). D-serine was also an encountered residue in the peptide chain of *Enterococcus gallinarum* (Vollmer et al., 2008). However, other D-amino acids are also naturally produced by bacteria and accumulate in high concentrations (mM) in the supernatant of medium during stationary phase (Lam et al., 2009). The release of D-amino acids into the environment is thought to regulate interspecies events, as D-peptides, also described as

peptide antibiotics are commonly produced by bacteria as protective measurement against other bacteria (Bodanszky and Perlman, 1969). *Bacillus* species incorporate D-amino acids into surfactin, which is a very potent antibiotic that disintegrates bacterial cell membranes, causing subsequent lysis of the cell (Marahiel et al., 1993). When D- amino acids accumulate within the cell, this causes changes in the composition of peptidoglycan, as D-Met for example is incorporated into *V. choleraes* muropeptides (Cava et al., 2011), which affects the general amount and strength of the peptidoglycan. This is a hint that D-amino acids are used as adaptation tool to cope with environmental changes, as they are incorporated during stationary phase, and provide the possibility of altering the peptidoglycan layer. At first, it was also assumed that D-amino acids cause biofilm disassembly (Kolodkin-Gal et al., 2010), but later studies could show that this effect is caused only by the toxic effects that D-amino acids have on protein biosynthesis (Leiman et al., 2013). Specifically, D-amino acids cause problems by false integration into proteins, as the laboratory wild type strain 168 carries a mutation in *dtd*, encoding for the D-tyrosyl-tRNA deacylase that would otherwise prevent the misincorporation from happening. It is tempting to speculate that D-amino acids enter the cell in the same way as L-amino acids do, as their structural similarity could fit into the same binding pocket of the uptake transporter. A case of this being true was reported for L and D-alanine, which both enter the cell via AlaP, the general alanine uptake transporter in *B. subtilis* (Sidiq et al., 2021). This principle was studied in chapter 4 of this thesis, using D-asparagine to trigger suppressor mutant formation. Although high concentrations of D-amino acids have harmful effects, a few bacterial species are also able to metabolize D-amino acids and use them as carbon or nitrogen source, as was reported for several Actinobacteria and Proteobacteria inhabiting extreme environments such as deep subsurfaces or arctic ice (Radkov et al., 2016; Wang et al., 2021). The degradation of D-amino acids takes place in two ways: The direct oxidation of the D-amino acid to a α-keto acid or the transformation of a D-amino acid into its L counterpart by a racemase. Although oxidation was found in multiple bacteria and even the common model organism *E. coli* (Olsiewski et al., 1980), it was not reported to take place in *B. subtilis*. A strain of *Halomonas sp.* from the hadal trench is able to utilize a broad range of D-amino acids, even preferring them over L-amino acids, by usage of a broad-spectrum D-amino acid dehydrogenase (Wang et al., 2021). The process of reverse racemization was also reported in deep sea microorganisms, such as the marine archeon *Methanococcus maripaludis*, but also in the yeast *Schizosaccharomyces pombe*. Both show rapid utilization of D-alanine via quick racemization (Zhang and Sun, 2014). AlrA, the alanine racemase is also present in *B. subtilis* and is even essential (Kobayashi et al., 2003), as it provides the cells with a constant supply of D-alanine, which is needed to build up the cell wall (Sidiq et al., 2021). *B. subtilis* also possesses the non-essential broad range racemase RacX, which catalyzes the reaction of 16 different L-amino acids to their D- enantiomer (Miyamoto et al., 2017). It is speculated, that these D-amino acids might play a role in peptidoglycan remodeling, or counteract biofilm formation (She et al.,

2015). Overall, D-amino acids are still an underresearched topic and could provide interesting insights into metabolic pathways, interspecies regulation and general transport mechanisms.

2.3.3. Amino acids become toxic in a strain lacking c-di-AMP

Cyclic dimeric adenosine 3'-5'-monophosphate (c-di-AMP) is a second messenger molecule in *B. subtilis*, which carries out numerous regulatory processes (Commichau et al., 2015b). It controls cell wall as well as potassium homeostasis and also ensures the integrity of the DNA (Gundlach et al., 2015). It is the only essential second messenger in *B. subtilis*, and was found to be essential in other bacterial species as well (Song et al., 2005; Glass et al., 2006). Although being essential, intracellular accumulation of c-di-AMP is lethal to the cells, which earned it the description "essential poison" (Gundlach et al., 2015; Huynh et al., 2015). This illustrates that the c-di-AMP levels within the bacterial cell have to be tightly regulated at all times. C-di-AMP is synthesized by three separate diadenylate-cyclases in *B. subtilis* from 2 molecules of ATP (Mehne et al., 2013). Of these, DisA and CdaA are responsible for c-di-AMP synthesis under standard conditions, while CdaS is the sporulation-specific diadenylate cyclase (Nicolas et al., 2012). A deletion mutant of all three synthesis genes is labelled as Δ*dac* (deletion of diadenylate cyclases) mutant. Such a mutant is not viable on complex medium anymore and thus bacteria require a specified minimal medium (MSSM), with sodium as buffer instead of potassium, to grow. This is required, as 9 out of the 18 target proteins of c-di-AMP are involved in potassium homeostasis (Stülke and Krüger, 2020), which does not function properly in its absence. Of these, c-di-AMP directly binds to all the known potassium importers KimA, KtrA and KtrC (Stülke and Krüger, 2020), controlling their activity at gene expression and general activity levels (Gundlach et al., 2019). Without c-di-AMP the import of potassium happens unregulated which causes the potassium concentration to be too high for the cells. Still, low concentrations of potassium need to be included in the medium, as no living cell is able to function completely without potassium. The Δ*dac* strain of *B. subtilis* uses potassium to balance out the negative charge of the DNA. It was also shown that positively charged amino acids, like arginine, ornithine or citrulline could rescue a strain growing under extreme potassium limitation (Gundlach et al., 2017a) and therefore potentially overtake its role in balancing the intracellular charges. In the Δ*dac* strain of *B. subtilis* however, several amino acids were shown to be harmful (Fig. 5) (Herzberg et al., 2023; Krüger et al., 2021). This results from the increased glutamate production that occurs in many cases if amino acids are supplemented to the medium, as the degradation pathways of plenty amino acids flow into glutamate production. This then causes an affinity change in the potassium uptake transporter KtrCD, causing more potassium to be imported into the cell (Krüger et al., 2020). Histidine, another positively charged amino acid is also toxic to the Δ*dac* strain of *B. subtilis*. When subjected to toxic levels of histidine, suppressors respond with enhanced export of histidine, as illustrated in chapter 3 of this thesis (Meißner et al., 2022). The other

mutations occuring under amino acid pressure often directly affect the potassium importers KtrCD and KimA (Gundlach et al., 2019), as well as essential genes, like AccA and FusA (Krüger et al., 2021; Herzberg et al., 2023). This suggests a general downregulation of metabolic processes at a point where the cell cannot deal with the overflow of amino acids anymore. Even the compatible solute proline, which is normally critical for cellular defense against high osmolarity was shown to cause toxic effects in the Δ*dac* strain, which illustrates the general sensitivity of the c-di-AMP free strain once more. We used this sensitivity in several experiments, where we supplemented the medium with various amino acids to trigger suppressor mutant formation.

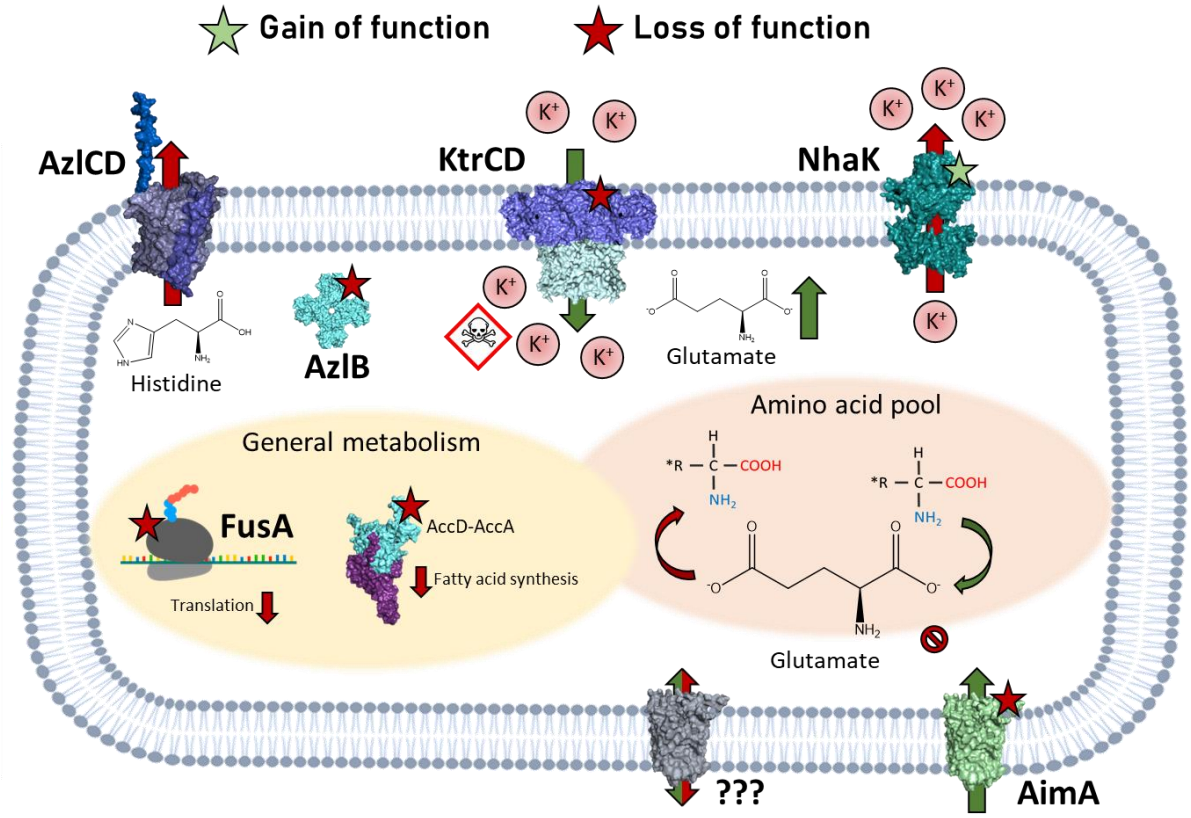

Figure 5 Effects of amino acids on a c-di-AMP free strain of *B. subtilis,* taken from (Herzberg et al., 2023). Amino acids, primarily glutamate, make up by far the largest fraction of the metabolite content of *B. subtilis* cells. Without c-di-AMP however, glutamate becomes toxic, as it causes an affinity change of the potassium-channel KtrCD, leading to an increased influx of potassium ions, which are harmful to the cell. *B. subtilis* responds to glutamate stress cia mutations in AimA, the major glutamate transporter and KtrCD itself (Krüger et al., 2021). Higher concentrations of glutamate cause downregulations in general metabolism. Here, the essential genes *fusA* (involved in translation) and *accA* (involved in fatty acid biosynthesis) are affected. Furthermore, *nhaK* also acquires a gain-of-function mutation resulting in enhanced potassium export under glutamate stress. Other amino acids feed into glutamate production and therefore might also be harmful in a c-di-AMP free setting. Stressing the cells with histidine leads to mutations in the transcriptional repressor AzlB, which causes an overexpression of the bipartite exporter AzlCD. This causes histidine to be exported out of the cell with higher efficiency (Meißner et al., 2022).

2.3.4. Asparagine becomes toxic in a strain lacking the asparaginases AnsA and AnsZ

L-Asparagine is a proteinogenic amino acid that is formed by three separate synthetases in *B. subtilis*: AsnO, AsnB and AsnH, which all hydrolyze glutamine to form asparagine. AsnB was found to be essential in *B. subtilis*, as it carries out a secondary function; the amidation of meso-diaminopimelic (mDAP) acid in peptidoglycan (Dajkovic et al., 2017). A knockout only is possible if excess Mg^{2+} is provided to the cells, as this helps keeping up the balance between PG synthesis and hydrolysis. Asparagine is mainly degraded to aspartate and ammonia by the two asparaginases AnsA and AnsZ. The presence of two asparaginases is shared with *E. coli*, which also possesses two asparaginases, a low affinity and a high affinity enzyme, respectively (Srikhanta et al., 2013). The subsequent reaction by the aspartase AnsB degrades aspartate to fumarate and ammonia, thus feeding into the TCA. This illustrates the constant interplay between the intracellular concentration of asparagine and aspartate, as their degradation enzymes are also co-transcribed in the *ansAB-mleNA* operon. In a bacterial cell, the general concentration of aspartate is about ten times higher than the asparagine concentration (Bennett et al., 2009), which underlines the importance of a steady supply of aspartate. AspB, the aspartate synthase of *B. subtilis* is also needed to maintain synthesis of asparagine, as the asparagine synthases need glutamine as well as aspartate as starting substance. Therefore, a knockout strain of *aspB* is auxotrophic for both, asparagine and aspartate (Zhao et al., 2018). In contrast to asparagine, aspartate is involved in many intracellular reactions, as it is involved in β-alanine production via PanD (Deng et al., 2015) and is also a precursor for pyrimidine and peptidoglycan biosynthesis (Brabson et al., 1985; Zhao et al., 2018) Apart from self-synthesis, *B. subtilis* can also take up aspartate via the main aspartate transporter GltT (Zaprasis et al., 2015), or potentially via YveA (Lorca et al., 2003), which is a paralog to the general amino acid importer AimA. Asparagine uptake however is still unexplored in *B. subtilis*, but might be shared between multiple transporters, as BLAST results of *S. typhimurium* AnsP yielded 6 homologs with a sequence identity of at least 35% (AlaP 41%, YdgF 40%, AapA 39%, YbxG 39%, GabP 37%, YbgF 35%). This multitude of transporters might differ from the findings in *S. enterica* and *E. coli*, which both seem to have a major asparagine transporter (Srikhanta et al., 2013; Jennings et al., 1995). In *E. coli*, this transporter AnsP is less expressed under nutrient rich conditions, as it is repressed by the global regulator protein Fis (Srikhanta et al., 2013). A problem complicating the analysis of transporters is the general conserved structure of the transporters, while simultaneously possessing a low substrate-specificity. We researched asparagine uptake via the chiral enantiomer D-asparagine. D-Asparagine has no known uptake transporter in *B. subtilis* and could act as a toxic analogon to L-asparagine. We also created genotypes sensitive to L-Asn pressure. A knockout of the two asparaginase genes *ansA* and *ansZ* in *B. subtilis* results in an asparagine sensitive strain. The reason for the toxicity of asparagine is not clear yet, but the balance between intracellular asparagine and aspartate concentration might be disrupted in the Δ*ansAB* Δ*ansZ* double knockout mutant. We

researched the cellular reactions to asparagine stress in *B. subtilis* via suppressor mutants obtained from D-asparagine and L-asparagine pressure likewise.

2.4. Strategies to identify novel amino acid transporters

In order to identify novel transporters in *B. subtilis*, multiple strategies were employed to yield insights into amino acid uptake. The first approach uses toxic analoga of amino acids, which cause adaptive reactions in the cell that are often located in transporters. This delivers a hint towards the actual substrate that is transported by the concerned protein. Another strategy involves the creation of genotypes, which are prone to amino acid stress, like a strain lacking c-di-AMP or degradation enzymes for a certain amino acid. Stressing such a strain with the L-variant of amino acids and researching the response of the cells towards such a stress event directly yields insights into metabolism. The analysis of a metabolic profile of the wild type strain compared to the transporter mutant further solidifies hypotheses about the transported substrate, as also done in (Meißner et al., 2022). Another classical approach is the utilization of radiolabelled metabolites, which can be tracked intracellularly and thus reveal uptake transporters (Westlake and Mackie, 1990). While being usually a good guiding point, analysis of the cluster of orthologous genes (Galperin et al., 2021) is difficult to utilize when researching amino acid transporter. Even though conserved proteins often carry out the same function, this principle often does not hold true in the case of amino acid transporters, as they are ambiguous in their binding substrates. Transposon sequencing could be a valid method in identification of amino acid transporters, although it would require an amino acid sensitive strain to start with. In *B. subtilis* this strain usually forms suppressors affecting the transporter, so this has been proven to be more useful in this regard. Cryo electron microscopy is a powerful tool that yields valuable insights into the binding pocket of transporters and thus also helps with the characterization of transport proteins. It helped understanding the properties of the potassium importers of *B. subtilis* to a substantial degree (Stautz et al., 2021). Lastly, modern protein interaction tools, such as AlphaFold-Multimer might reveal novel interactions of uncharacterized proteins and also reveal binding partners for these proteins (Evans et al., 2021).

2.5. Aim of this work

This study aims to enhance understanding of general amino acid metabolism in *B. subtilis*, specifically touncover the metabolic pathways of the uncharacterized amino acids histidine and asparagine. To elucidate the transport mechanisms of these amino acids, different strategies were employed in order to obtain suppressor mutants. Therefore, amino acid sensitive genotypes were grown under amino acid stress. A conceptual approach was utilized as well, to research the possibility of D-amino acids entering the cells via the same transporters as L-amino acids. The isolated suppressors were analyzed in diverse growth experiments and metabolomics evaluations as well as expression analysis of the transporters. This work elucidates toxic amino acids in wild type, Δ*dac* and Δ*ansAB* Δ*ansZ* background and discusses the reasons for the amino acid toxicity.

Chapter 3 – How to deal with toxic amino acids: The bipartite AzlCD Complex exports Histidine in *Bacillus subtilis*

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The results described in this chapter were originally published in the *Journal of Bacteriology* (doi:10.1128/jb.00353-22)

ABSTRACT

The Gram-positive model bacterium *Bacillus subtilis* can use several amino acids as a source of carbon and nitrogen. However, some amino acids inhibit the growth of this bacterium. This amino acid toxicity is often enhanced in strains lacking the second messenger cyclic di-AMP. We observed that the presence of histidine is also toxic for a *B. subtilis* strain that lacks all three c-di-AMP synthesizing enzymes. However, suppressor mutants emerged, and whole genome sequencing revealed mutations in the *azlB* gene encoding the repressor of the *azl* operon. This operon encodes an exporter and an importer for branched-chain amino acids. The suppressor mutations result in overexpression of the *azl* operon. Deletion of the *azlCD* genes encoding the branched-chain amino acid exporter restored the toxicity of histidine indicating that this exporter is required for histidine export and resistance to otherwise toxic levels of the amino acid. The higher abundance of the amino acid exporter AzlCD increased the extracellular concentration of histidine, thus confirming the new function of AzlCD as a histidine exporter. Unexpectedly, AzlB-mediated repression of the operon remains active even in the presence of amino acids suggesting that expression of the *azl* operon requires mutational inactivation of AzlB.

IMPORTANCE

Amino acids are building blocks for protein biosynthesis in each living cell. However, due to their reactivity as well as the similarity between several amino amino acids, they may also be involved in harmful reactions or in non-cognate interactions and thus be toxic. *Bacillus subtilis* can deal with otherwise toxic histidine by overexpressing a bipartite amino acid exporter AzlCD. Although encoded in an operon that also contains a gene for an amino acid importer, the corresponding genes are not expressed, irrespective of the availability or not of amino acids in the medium. This suggests that the *azl* operon is a last resort to deal with histidine stress that can be expressed due to mutational inactivation of the cognate repressor, AzlB.

KEYWORDS

Bacillus subtilis, cyclic di-AMP, amino acid export, histidine, amino acid toxicity, silent genes

INTRODUCTION

Amino acids are the essential building blocks for protein biosynthesis and many other cellular components. Cells can acquire amino acids by uptake from the environment, by degradation of external peptides or proteins, or by *de novo* biosynthesis. Many bacteria such as the model organisms *Escherichia coli* and *Bacillus subtilis* can use all three possibilities for amino acid acquisition. Although amino acids are essential for growth, they can be toxic due to misloading of tRNAs resulting in misincorporation into proteins and from their high reactivity. Moreover, many amino acids are chemically very similar to each other, and one amino acid that is available in excess may competitively inhibit the biosynthetic pathway(s) of similar amino acids by binding to the corresponding enzymes (de Lorenzo et al., 2015).

We are interested in the identification of the components that allow life of a simple minimal cell and in the construction of such cells based on the model bacterium *B. subtilis* (Commichau et al., 2013; Reuß et al., 2016). Such minimal organisms are not only important to get a comprehensive understanding of the requirements of cellular life but they are also important workhouses in biotechnological and biomedical applications. Indeed, minimal organisms have been proven to be superior to conventionally constructed strains in the production and secretion of difficult proteins and lantibiotics (Aguilar Suárez et al., 2019; van Tilburg et al., 2020; Michalik et al., 2021).For *B. subtilis*, the pathways for all amino acid biosyntheses have been completely elucidated. In contrast, the knowledge about amino acid transport is far from being complete as for several amino acids no transporter has been identified so far. This knowledge is important for the construction of genome-reduced strains that may be designed to grow in complex or minimal medium and thus require the complete set of uptake or biosynthetic enzymes, respectively. Moreover, some amino acids such as glutamate are toxic for *B. subtilis* even at the concentrations present in standard complex medium if the catabolizing enzymes, e.g. glutamate dehydrogenase, are absent (Commichau et al., 2008; Gunka et al., 2012). Thus, a complete understanding of all components involved in cellular amino acid homeostasis is required for the succsessful generation of minimal organisms.

Amino acid toxicity is not only relevant for the design of minimal genomes but it is also an important tool for the identification of components involved in amino acid metabolism. While some amino acids such as serine or threonine are toxic already for wild type strains (de Lorenzo et al., 2015; Belitsky, 2015; Klewing et al., 2020), others are well tolerated. In this case, the corresponding D-amino acids, amino acid analogs, or structually similar metabolites may act as anti-metabolites that inhibit normal cellular metabolism and thus growth of the bacteria. The application of toxic amino acids or of similar compounds to a bacterial growth medium will inhibit growth but will also result in the acquisition of suppressor mutations that allow the cells to resolve the issue of amino acid toxicity. Often, such mutations affect uptake systems and prevent the uptake of the toxic amino acid or its

analogues. In this way, transporters for threonine, proline, alanine, serine, and glutamate as well as for the anti-metabolites 4-hydroxy-L-threonine and glyphosate have been identified in *B. subtilis* (Belitsky, 2015; Klewing et al., 2020; Commichau et al., 2015a; Zaprasis et al., 2014; Sidiq et al., 2021; Krüger et al., 2021; Wicke et al., 2019). A second way to achieve resistance against toxic amino acids is the activation of export mechanisms. This has been reported in the cases of 4-azaleucine and glutamate (Krüger et al., 2021; Ward and Zahler, 1973). Third, suppressor mutations may facilitate detoxification of the toxic amino acid by degradation or modification to a non-toxic metabolite as observed for glutamate and serine (Commichau et al., 2008; Klewing et al., 2020; Krüger et al., 2021; Belitsky and Sonenshein, 1998). Forth, the protein target of the toxic metabolite may be modified in a way that it becomes resistant (Comai et al., 1983). Finally, the bacteria can escape inactivation by increased expression of the target protein as has been reported for serine and the anti-metabolite glyphosate (Klewing et al., 2020; Wicke et al., 2019).

Recently, it has been shown that the sensitivity of *B. subtilis* to glutamate is strongly enhanced if the bacteria are unable to produce the second messenger cyclic di-AMP (c-di-AMP) (Krüger et al., 2021). This second messenger is essential for growth of *B. subtilis* on complex medium, and it is toxic upon intracellular accumulation (Stülke and Krüger, 2020). Both essentiality and toxicity are mainly a result of the central role of c-di-AMP in potassium homeostasis. The second messenger prevents the intracellular accumulation of potassium by inhibiting potassium import and by stimulating potassium export. Thus, the intracellular potassium concentration is kept within a narrow range (Stülke and Krüger, 2020; Gundlach et al., 2019). The presence of high potassium concentrations in a strain lacking c-di-AMP results in the activation of potassium export by the acquisition of mutations in a sodium/H^+ antiporter. These mutations change the specificity of the antiporter towards potassium (Gundlach et al., 2017b). Even though none of the known targets of c-di-AMP is directly involved in glutamate homeostasis, glutamate is as toxic as potassium to the mutant lacking all diadenylate cyclases that would synthesize c-di-AMP. This can be explained by the fact that glutamate activates the low-affinity potassium channel KtrCD by strongly increasing the affinity of this channel. Thus, even the very low potassium concentration, which must be present even for the growth of this strain, become toxic due to the high affinity of KtrCD for potassium in the presence of glutamate (Krüger et al., 2020). Accordingly, the Δ*dac* strain lacking c-di-AMP acquires mutations that reduce potassium uptake if propagated in the presence of glutamate. In addition, the bacteria usually acquire additional mutations that interfere with glutamate homeostasis by reducing uptake, facilitating export, or allowing degradation of the amino acid (Krüger et al., 2021).

In this study, we were interested in the control of histidine homeostasis. Histidine biosynthesis from ribose 5-phosphate requires ten enzymes (see http://subtiwiki.uni-goettingen.de/v4/category?id=SW.2.3.1.14; 23). The degradation of histidine to ammonia, glutamate,

and formamide involves a specific transporter, HutM, and four enzymes. The histidine transporter is induced in the presence of histidine, which is a typical feature for high-affinity transport systems (Wray and Fisher, 1994; Bender, 2012). Usually, high-affinity transporters are used for catabolic pathways to use an amino acid as carbon or nitrogen source. In contrast, constitutively expressed low-affinity transporters are used to import the amino acid from complex medium for protein biosynthesis. In many cases, both, low- and high-affinity amino acid transporters, are encoded in the genome of *B. subtilis*, and they are expressed depending on the pyhsiological conditions. However, no low-affinity histidine transporter has been identified so far. Histidine degradation yields intracellular glutamate, which is toxic for mutants lacking c-di-AMP (Krüger et al., 2021) due to the activation of the potassium channel KtrCD (Krüger et al., 2020). We thus expected that the strain lacking c-di-AMP has a similar sensitivity against histidine as it is against glutamate. We made use of this sensitivity to the degradation product glutamate to get further insights into the components that contribute to histidine homeostasis in *B. subtilis*. Our study revealed that mutational activation of an export system is the major mechanism to achieve resistance to histidine.

RESULTS

Histidine is toxic to a *B. subtilis* strain lacking c-di-AMP, and mutations in the *azlB* gene overcome the toxicity. Some amino acids such as serine and threonine are toxic for *B. subtilis*. In the case of glutamate, toxicity becomes visible in the absence of a degradation pathway or if the bacteria are unable to form the second messenger c-di-AMP (Krüger et al., 2021). Here, we tested growth of a wild type strain (168) of *B. subtilis* and of an isogenic strain that had all three genes encoding diadenylate cyclases deleted (Δ*dac*, GP2222) (Gundlach et al., 2017b) in the presence of histidine. As shown in Fig. 1, growth of the *B. subtilis* wild type strain 168 is not affected by histidine concentrations up to 10 mM. At a higher concentration of 20 mM, growth was inhibited. In contrast, histidine is highly toxic for *B. subtilis* GP2222 even at very low histidine concentrations (see Fig. 1).

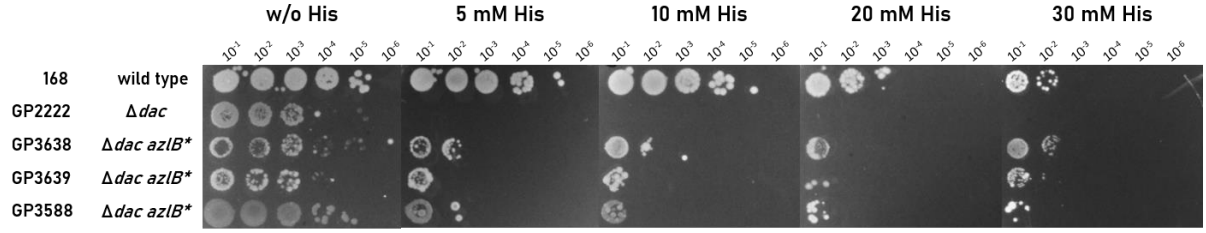

Figure 6 The isolated suppressors are resistant to histidine stress. Growth of *B. subtilis* suppressor mutants (GP3638, GP3639 and GP3588) in the presence of histidine. All suppressors carry different mutations in the *azlB* gene (see Appendix). Cells were harvested and washed, and the OD_{600} was adjusted to 1.0. Serial dilutions were added dropwise to MSSM minimal plates with the indicated histidine concentration. Plates were incubated at 42°C for 48 h.

We observed that larger colonies rapidly appeared. It is likely that these larger colonies were formed bysuppressor mutants that were resistant to histidine in the medium. We hypothesized that mutations could affect uptake systems for histidine as already observed for glutamate, serine, or threonine (9, 10, 14). Indeed, we were able to indentify mutations in two isolates by whole genome sequencing. However, to our surprise, the mutations did not cover known or putative amino acid transporters of *B. subtilis* (23). In contrast, we observed mutations in the *azlB* gene, which encodes a Lrp-type transcription repressor that controls the expression of a branched chain amino acid exporter (AzlCD) and a branched chain amino acid importer (BrnQ) (9, 26). Strain GP3638 carried an amino acid substitution in AzlB (Asn24 Ser). In the second strain, GP3639, we found an eight basepair insertion (CATTAATG) after the 37th basepair of the coding sequence that results in a frameshift and thus prevents the expression of a functional AzlB protein. As the *azlB* gene seemed to be a hotspot of mutations in histidine-resistant suppressor mutants, we determined the sequence of this gene in four additional mutants. In each case, mutations were present in the *azlB* gene, either amino acid substitutions, Asn24 Ser as in GP3638, Ile31 Met, or frameshift mutations. Since the frameshift mutations prevent the formation of functional AzlB proteins, it seemed likely that the amino acid substitutions also resulted in inactive proteins. Indeed both the N24S and the I31M mutations affect the DNA-binding helix-turn-helix motif of AzlB.

As mentioned above, growth of the wild type strain 168 was inhibited above 20 mM histidine. Therefore, we tested the growth of our suppressor mutants in the absence and presence of histidine. While all mutants were viable at 5 mM histidine, they were still inhibited at a concentration of 30 mM (see Fig. 1). However, when suppressor mutants originally isolated at 15 mM histidine were transferred to 30 mM histidine, suppressor mutants appeared again. One of these mutants (GP3588) was subjected to whole genome sequencing. In coherence with our previously isolated mutants, we found a frameshift mutation in *azlB* highlighting the importance of *azlB* inactivation for the adaptation of the *B. subtilis* strain lacking c-di-AMP to the presence of histidine. Moreover, we found three additional mutations at a histidine concentration of 30 mM. Both the main potassium transporter KimA and the KtrD membrane subunit of the low-affinity potassium channel KtrCD (Gundlach et al., 2017b; Holtmann et al., 2003) were inactivated due to frameshift mutations. In addition, the high affinity glutamate transporter GltT (Krüger et al., 2021; Zaprasis et al., 2015) carried a substitution of Thr-342 to a Pro residue. It is known that KtrCD is converted to a high-affinity potassium channel in the presence of glutamate (Krüger et al., 2020), suggesting that glutamate as the product of histidine utilization causes activation of KtrCD. Moreover, small amounts of glutamate that are exported from the cell may be re-imported by GltT thus, again, contributing to the activation of KtrCD. This activation of KtrCD as well as the activity of KimA contribute to potassium toxicity that can only be bypassed by inactivation of the major potassium uptake systems.

Histidine toxicity in the Δ*dac* mutant GP2222 might be caused by the formation of glutamate that triggers toxic glutamate accumulation, by toxicity of histidine due to its chemical reactivity, or by a combination of both. The identification of *kimA* and *ktrD* mutants in the suppressor isolated at the elevated histidine concentration suggests that potassium toxicity really can become a problem for the bacteria. However, we never identified suppressor mutants that were affected in the histidine degradaton pathway thus avoiding the problem of intracellular glutamate formation. To test the role of histidine degradation for the acquisition of resistance to histidine, we deleted the *hutH* gene encoding histidase, the first gene of the catabolic pathway in a wild type and a Δ*dac* mutant strain. The set of four isogenic strains was tested for growth on minimal medium in the absence of histidine and in the presence of 5 mM, 15 mM, 25 mM, and 35 mM histidine. While all strains grew well in the absence of histidine, growth was inhibited at histidine concentrations exceeding 15 mM and 5 mM for the wild type and the Δ*dac* mutant, respectively. The inactivation of the histidine degradative pathway (*hutH* mutation) did not affect growth in either of the genetic backgrounds (data not shown). Thus, growth inhibition of the Δ*dac* mutant by histidine seems to result from a combination of (i) its own chemical reactivity as has also been observed for *E. coli* (Nagao et al., 2018) and (ii) from its conversion to glutamate that triggers toxic potassium accumulation in this strain.

Taken together, our results demonstrate that the Lrp-type repressor protein AzlB plays a major role in the adaptation of *B. subtilis* lacking c-di-AMP to high levels of histidine. At even higher concentration of histidine, the degradation product glutamate induces the uptake of potassium, which is known to be toxic to strains that are unable to produce c-di-AMP (Gundlach et al., 2019; Gundlach et al., 2017b; Krüger et al., 2020).

Suppressor mutants exhibit increased expression of the *azlBCD-brnQ* operon.

To test the effect of the *azlB* mutations on the expression of the *azlBCD-brnQ* operon, we analyzed the transcripts of the operon by a Northern blot analysis. For this purpose, we cultivated the wild type strain 168, the Δ*dac* mutant GP2222 and two suppressor mutants GP3638 (AzlB-Asn24 Ser) and GP3639 (frameshift in AzlB) in MSSM minimal medium, isolated the RNA, and performed Northern blot experiments using a riboprobe complementary to *azlC* to detect the specific mRNA(s). Based on the known transcript sizes of the *B. subtilis* glycolytic *gapA* operon (Meinken et al., 2003), we estimated the sizes of the transcripts of the *azl* operon. As shown in Fig. 2A, expression of the operon could not be detected in the wild type strain 168 and in the Δ*dac* mutant GP2222. Signals corresponding to transcripts of about 1,100, 1,500, 3,300 and 5,100 nucleotides were only visible in the two suppressor mutants carrying the *azlB* mutations. This result indicates that only inactive *azlB* allows expression of the *azl* operon and led to high expression levels. The presence of multiple transcripts suggests internal transcription signals and/or mRNA processing events.

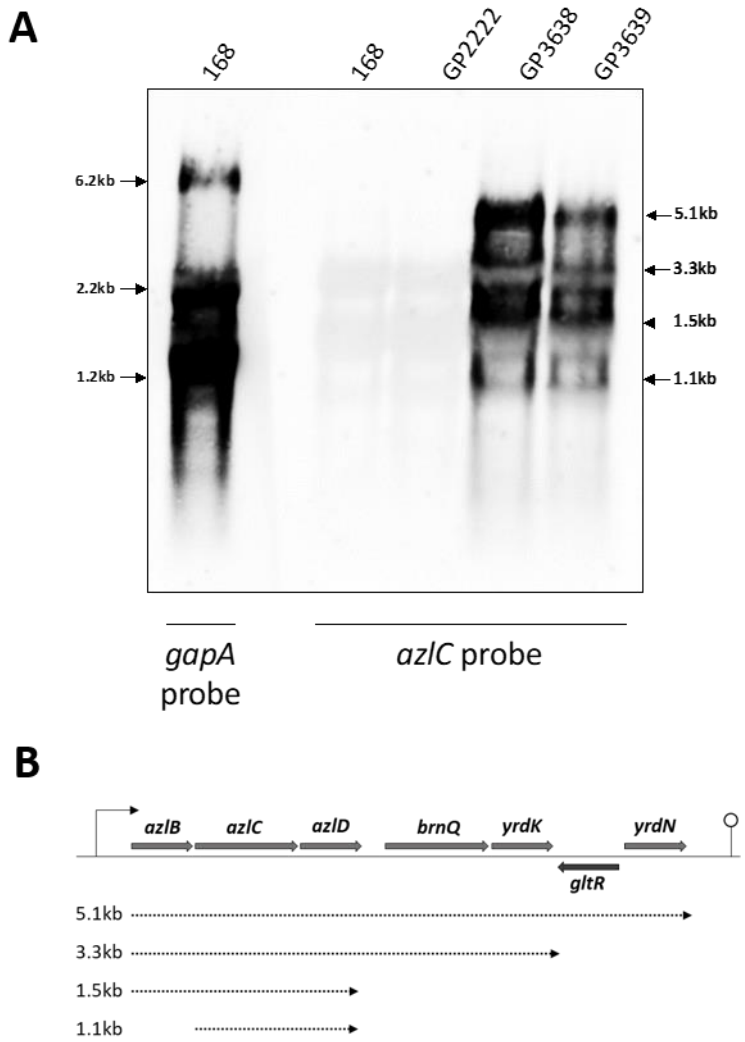

Figure 7 AzlCD is strongly overexpressed in the histidine suppressors. **A.** Northern Blot analysis to test the expression levels of the *azl* operon in the suppressor mutants GP3638 and GP3639. The RNA was isolated from MSSM minimal medium during exponential growth phase. The *gapA* probe was used together with wild type RNA as control to estimate band sizes and strength, as it is strongly expressed under normal conditions. **B.** Transcriptional organization of the *azl* operon. The length of the individual transcripts is indicated by the arrows. The position of the transcripts that are smaller than the complete operon mRNA is deduced from the determined transcript sizes and the genomic arrangement. The bent arrow and the hairpin correspond to the positions of the transcriptional up- and downshifts, respectively.

So far, the inducer for the *azlBCD-brnQ* operon has not been identified. Since our results indicate that the operon is involved in the control of the histidine homeostasis, we wanted to test the activity of the *azlB* promoter under different conditions. For this purpose, we fused the *azlB* promoter region to a promoterless *lacZ* gene encoding β-galactosidase and integrated this *azlB-lacZ* fusion into the *B. subtilis* genome. According to a genome-wide transcriptome analysis (Nicolas et al., 2012), the promoter of the operon is located in front of the upstream *yrdF* gene. However, the same study indicated the presence of an mRNA upshift in front of the *azlB* gene. Similarly, Belitsky *et al.,* identified

promoter activity immediately upstream of *azlB* (Belitsky et al., 1997). To clarify this issue, we also constructed and tested an *yrdF-lacZ* fusion. The strains carrying the *azlB-lacZ* and *yrdF-lacZ* fusions were cultivated in C-Glc minimal medium in the absence or presence of different amino acids as potential inducers. As shown in table 2, both the upstream regions of *yrdF* and *azlB* had only very minor promoter activity.

Table 2. Activity of the *azlB* promoter

Strain	Relevant genotype	**Units of β-galactosidase per μg of protein** Addition to C-Glc minimal medium				
		-	Ile	Pro	His	CAA
GP314	*pgi-lacZ*	48 ± 3	ND[1]	ND	ND	ND
GP3612	*azlB-lacZ*	4 ± 0.6	3 ± 0.7	2 ± 0.2	4 ± 0.6	7 ± 1.5
GP3614	*azlB-lacZ* Δ*azlB*	26 ± 5	25 ± 2	31 ± 4	31 ± 4	49 ± 7
GP3617	*azlB-lacZ* Δ*gltR*	3 ± 0.3	ND	ND	ND	5 ± 0.1
GP3611	*yrdF-lacZ*	3 ± 0.7	4 ± 1	2 ± 0.3	5 ± 2	ND
GP3613	*yrdF-lacZ* Δ*azlB*	3 ± 0.3	3 ± 0.6	3 ± 0.1	3 ± 0.4	ND

[1] ND, not determined

As a control, we used the moderately expressed promoter of the *pgi* gene encoding phosphoglucose isomerase. This promoter yielded a ten-fold higher β-galactosidase activity as compared to the *yrdF* and *azlB* promoters. In addition, the activity of the *yrdF* and *azlB* promoters was not induced by any of the tested amino acids, including histidine. Therefore, we also tested casein hydrolysate, a mixture of amino acids. Again, no induction was observed for both promoters. However, deletion of the *azlB* gene resulted in an about seven-fold increase of the activity of the *azlB* promoter (see GP3614 vs. GP3612) whereas the *yrdF* promoter was not affected. Moreover, GltR, a LysR family transcription factor of so far unknown function, is encoded downstream of the *brnQ* gene (32). We therefore considered the possibility that GltR might play a role in the control of the *azl* operon. However, deletion of the *gltR* gene did not affect the activity of the *azlB* promoter.

Taken together, our data confirm that AzlB is the transcriptional repressor of the *azl* operon. The *azlB* gene is the first gene of the operon (see Fig. 2B). Moreover, our results demonstrate that the transcriptional regulation by AzlB is not affected by any individual amino acid or a mixure of them, even though the operon encodes exporters and importers for amino acids. Only the loss of a functional AzlB repressor allows the expression of the *azl* operon (see Discussion).

Resistance to histidine depends on the AzlCD amino acid exporter.

So far, we have established that the suppressor mutants have mutations in AzlB that increase expression of the *azl* operon, which confers resistance to histidine. In addition to the promoter-proximal repressor gene *azlB*, this operon encodes the AzlC and AzlD subunits of a bipartite amino acid exporter and the branched-chain amino acid transporter BrnQ as well as the YrdK protein of unknown function and the putative 4-oxalocrotonate tautomerase YrdN. Since overexpression of AzlCD was also responsible for the resistance of *B. subtilis* to azaleucine (Belitsky et al., 1997), it seemed most plausible that this transporter is also involved in histidine resistance. To test this hypothesis, we constructed two sets of isogenic strains that differed in the *azl* operon in the background of the wildtype 168 and in the background of the Δ*dac* mutant GP2222. First, we compared growth of the wild type, the *azlB* mutant GP3600, and the *azlBCD* mutant GP3601. As shown in Fig. 3A, the wild type strain was sensitive to the presence of 15 mM histidine in the medium, whereas the isogenic *azlB* mutant that exhibits overexpression of AzlCD was resistant. However, the additional deletion of the *azlCD* genes in GP3601 restored the sensitivity to histidine, indicating that the increased expression of the AzlCD amino acid exporter is responsible for the acquired resistance to histidine. Similar results were obtained for the set of strains that are unable to synthesize c-di-AMP (Δ*dac*, Fig. 3B). Again, the strain lacking AzlB (GP3607) was resistant to high levels of histidine (20 mM), whereas the strain lacking the amino acid exporter AzlCD in addition to AzlB (GP3606) was as sensitive as the Δ*dac* mutant (GP2222) even at 5 mM histidine. Ectopic expression of the *azlCD* genes under the control of the constitutive *degQ36* promoter (Martin-Verstraete et al., 1994) in strain GP3642 that lacks the endogenous *azlBCD* operon partially restored the resistance to histidine up to a concentration of 5 mM. In contrast, expression of the AzlC component of the bipartite exporter alone had no effect (Fig. 3B, GP3643). Taken together, these data strongly suggest that the overexpression of the two-component amino acid exporter AzlCD as a result of the inactivation of AzlB is required for the resistance of *B. subtilis* to histidine.

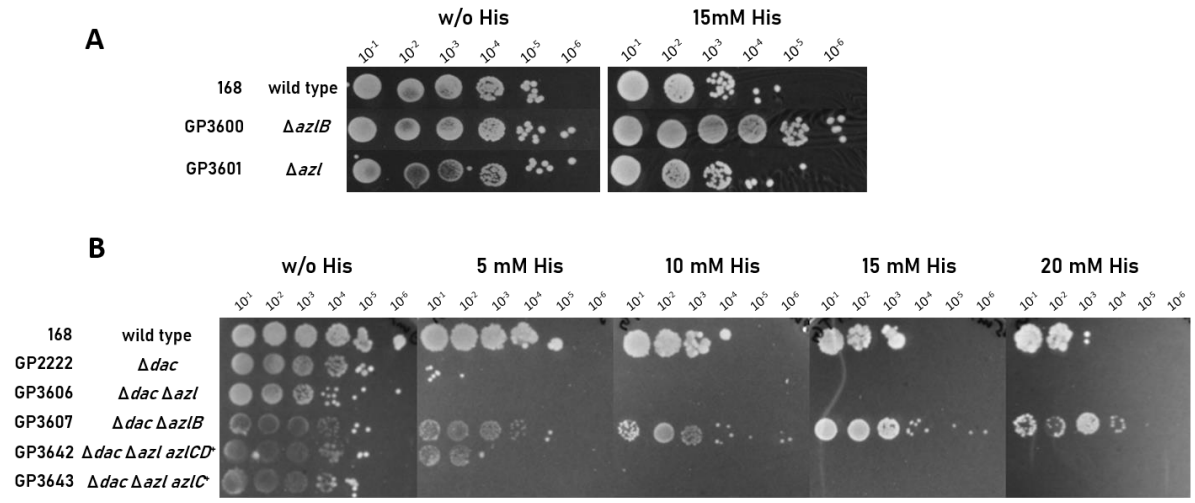

Figure 8 The *azlB* mutation confers resistance to histidine stress. A. Sensitivity of wild type *B. subtilis* (168) and the Δ*azlB* (GP3600) and Δ*azlBCD* (GP3601) mutants to histidine. Cells were grown in MSSM minimal medium to an OD_{600} of 1.0 and then diluted 10-fold to create dilutions ranging from 10^{-1} to 10^{-6}. The dilution series was dropped onto MSSM plates without and with (15 mM) histidine. The plates were incubated at 37°C for 48 h. **B.** Growth of Δ*dac* Δ*azl* (GP3606), Δ*dac* Δ*azlB* (GP3607) and Δ*dac* Δ*azl* complemented with *azlC* (GP3643) and *azlCD* (GP3642) respectively. Δ*azl* indicates a deletion of the *azlBCD* genes. Cells were grown as described above. The plates were incubated at 42°C for 48 h.

Overexpression of AzlCD results in enhanced histidine export.

AzlCD has previously been identified as an exporter for 4-azaleucine and was hypothesized to be an exporter for other branched chain amino acids (Belitsky et al., 1997). Our data suggest that the complex might also export histidine thus contributing to histidine resistance upon overexpression. To test this idea, we determined the relative intra- and extracellular histidine concentrations in the wild type strain 168, as well as in the isogenic *azlB*, *azlBCD*, and *azlCD* deletion mutants GP3600, GP3601, and GP3622, respectively, during growth in MSSM minimal medium. In this condition, *de novo* histidine biosynthesis is active, because MSSM minimal medium does not contain amino acids. Compared to the wild type, intracellular histidine levels decreased in the *azlB* mutant GP3600, thus confirming that higher AzlCD levels in this strain led to histidine export (Fig. 4A). Mutants lacking the amino acid exporter AzlCD had wild type-like histidine levels (Fig. 4A). In contrast, the extracellular histidine concentration was threefold higher in the *azlB* mutant whereas the strains lacking AzlCD have extracellular histidine levels that were comparable to the wild type strain (Fig 4 B). These results demonstrate that AzlCD which is overexpressed as a result of the *azlB* mutation, is involved in the control of histidine homeostasis. While the loss of AzlCD has no effect, which corresponds to the lack of expression in the wild type strain, its overexpression results in reduced and increased intra- and extracellular histidine levels, respectively. This suggests that AzlCD is an active histidine exporter.

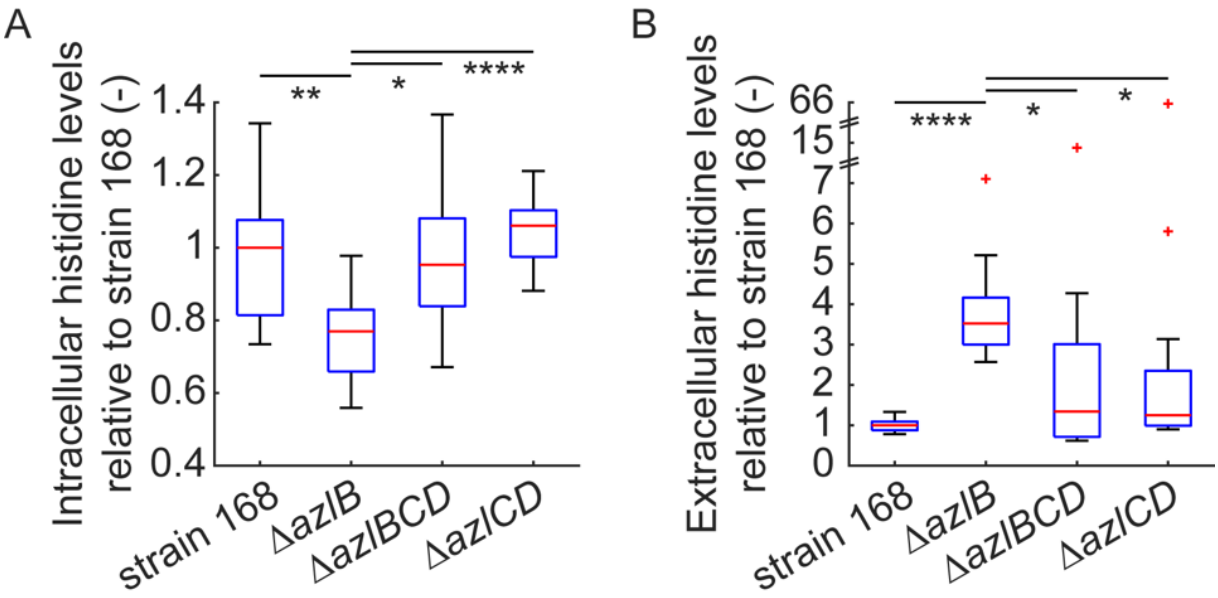

Figure 9 AzlCD is a histidine exporter in *B. subtilis*. Box-whisker plot of the intracellular (A) and extracellular (B) histidine levels of *B. subtilis* Δ*azlB*, Δ*azlBCD*, and Δ*azlCD* mutants relative to the wild type strain 168. The red lines indicate the median values of 12 biological replicates. The upper box edges show the 75th percentiles, the lower edges the 25th percentiles. The whiskers indicate the furthest points that are not considered outliers. The red crosses indicate outliers. Differences between indicated pairs of strains were tested for significance using a Wilcoxon rank sum test at a significance level α of 0.05. p-values < 0.05 were considered statistically significant. The stars indicate the orders of magnitude of the p-values: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

DISCUSSION

The results presented in this study demonstrate that histidine inhibits growth of *B. subtilis* as has already been shown for serine or threonine (Klewing et al., 2020; Lachowicz et al., 1996; Lamb and Bott, 1979a). Amino acid toxicity is often enhanced if *B. subtilis* is unable to produce the essential second messenger nucleotide c-di-AMP due to the activation of the potassium channel KtrCD by glutamate, the degradation product of many amino acids (Krüger et al., 2021; Krüger et al., 2020). This work shows that the increased sensitivity of a strain lacking c-di-AMP to amino acids is also valid for histidine.

Typically, suppressor screens using toxic amino acids, amino acids analogs or related anti-metabolites result in the identification of transporters, which have been inactivated in the suppressor mutants (Belitsky, 2015; Klewing et al., 2020; Commichau et al., 2015a; Zaprasis et al., 2014; Sidiq et al., 2021; Krüger et al., 2021; Wicke et al., 2019). While this is the predominant type of suppressor mutations, resistance to toxic amino acids and related molecules can also be achieved by the activation of degradation pathways (Klewing et al., 2020; Krüger et al., 2021), by the activation of export mechanisms (Krüger et al., 2021; Belitsky et al., 1997), or by modifying the target protein/ pathway in a way that it becomes insensitive to the presence of the otherwise toxic molecule. This was observed

for glyphosate resistance in *Salmonella typhimurium*, which can be achieved by mutations that render the target enzyme 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase insensitive to inhibition (Comai et al., 1983) as well as for serine toxicity in *B. subtilis*, which could be overcome by increased expression of the genes encoding the threonine biosynthetic pathway (Klewing et al., 2020). Studies about histidine toxicity in *E. coli* revealed that the amino acid enhances oxidative DNA damage (Nagao et al., 2018). Thus one might also expect suppressor mutations that prevent DNA damage. The exclusive isolation of *azlB* mutations that activate the expression of the AzlCD amino acid exporter suggests that all other mechanisms of suppression are more difficult to achieve for the *B. subtilis* cell.

The fact that we were unable to isolate a single suppressor mutant that had lost histidine uptake strongly suggests that *B. subtilis* possesses multiple histidine transporters. So far, only the HutM histidine transporter has been identified based on its similarity to known histidine transporters (Bender, 2012). It is tempting to speculate that the genome of *B. subtilis* encodes one or more low-affinity transporters for histidine. Indeed, *B. subtilis* encodes several homologs of the *Pseudomonas putida* histidine transporter HutT (Wirtz et al., 2021). These transporters all belong to the amino acid-polyamine-organocation (APC) superfamily of amino acid transporters. Four of them (AapA, AlaP, YbxG, and YdgF) share more than 40% sequence identity wih *P. putida* HutT, suggesting that these proteins have the same biological activity. Thus, the presence of multiple histidine uptake systems would prevent the rapid simultaneous inactivation of all these systems in suppressor mutants thus explaining that no transporter mutants were isolated.

Our data clearly demonstrate that the bipartite amino acid transporter AzlCD exports not only the leucine analog 4-azaleucine (Ward and Zahler, 1973), but also histidine. Corresponding bipartite systems that mediate the export of branched-chain amino acids have also been identified in *E. coli* and *Corynebacterium glutamicum* (Park et al., 2007; Kennerknecht et al., 2002). These exporters are members of the LIV-E class of transport proteins (Kennerknecht et al., 2002; Eggeling and Sahm, 2003). As in *B. subtilis*, these systems consist of a large (corresponding to AzlC) and a small subunit (corresponding to AzlD). While proteins homologous to AzlC are abundant in a wide range of bacteria, including most Actinobacteria and Firmicutes as well as many Proteobacteria, AzlD is conserved only in few bacteria. The other bacteria that possess a homolog of AzlC obviously have alternative small subunits. This is the case in *E. coli*, where the small YgaH subunit of the YgaZ/YgaH valine exporter is not similar to its counterparts in *B. subtilis* and *C. glutamicum*. We have also considered the possibility that the large subunit AzlC might be sufficient for histidine export; however, this is not the case (see Fig. 3B).

It is interesting to note that the AzlCD amino acid exporter is able to export multiple amino acids. Substrate promiscuity is a common feature in amino acid transport. In *B. subtilis*, the low affinity transporter AimA is the major transporter for glutamate and serine (Klewing et al., 2020; Krüger et al.,

2021). Similarly, the BcaP permease transports branched-chain amino acids, threonine and serine (9, 10, 11) and the GltT protein is involved in the uptake of aspartate, glutamate, and the antimetabolite glyphosate (Krüger et al., 2021; Wicke et al., 2019; Zaprasis et al., 2015). Thus, AzlCD is another example for the weak substrate specificity of amino acid transporters. It is tempting to speculate that AzlCD might be involved in the export of even other amino acids and related metabolites in *B. subtilis*.

Based on the chemical properties of each amino acid, it may be generally toxic, or only under specific conditions. Therefore, cells often have efficient degradation pathways to remove toxic compounds. This is the case for glutamate which is degraded by the glutamate dehydrogenases GudB or RocG (Commichau et al., 2008; Belitsky and Sonenshein, 1998). However, other amino acids become toxic only at very high concentrations or in very particular mutant backgrounds. This is the case for histidine which is toxic only at high concentrations for the *B. subtilis* wild type strain but already at low concentrations in a strain unable to form c-di-AMP. Similarly, the presence of amino acid analogs such as 4-azaleucine might be a rather exceptional event in natural environments. Still, *B. subtilis* is equipped to meet this challenge using the amino acid exporter AzlCD. Based on a global transcriptome analysis, the *azl* operon is barely expressed under a wide range of conditions, and no conditions that results in induction of the operon could be detected (Belitsky et al., 1997; Nicolas et al., 2012). Similarly, the putative arginine and lysine exporter YisU is not expressed under any of 104 studied conditions (Nicolas et al., 2012). The observation that the expression of the *azl* operon in the presence of toxic concentrations of histidine or 4-azaleucine is obviously not sufficient to provide resistance against these amino acids already suggested that none of these compounds acts as a molecular inducer for the *azl* operon. In agreement with previous results (Belitsky et al., 1997), we observed substantial expression of the operon only if the *azlB* gene encoding the repressor of the operon was deleted or inactivated due to the suppressor mutations. Even the presence of a mixture of amino acids derived from casamino acids did not result in the induction of the operon. As the functions of the operon seem to be related to amino acid export (AzlCD) and uptake (BrnQ), regulation by amino acid availability seemed to be most likely. However, the results from prior global and operon-specific transcription studies as well as our data suggest that the activity of AzlB is not controlled by amino acids even though the protein belongs to Lrp family of leucine-responsive regulatory proteins (Brinkman et al., 2003). It is tempting to speculate that AzlB has lost the ability to interact with amino acid-related effector molecules, but that expression of the operon can rapidly be activated by the acquisition of mutations that inactivate AzlB. Alternatively, AzlB might respond to a yet unknown signal and then allow induction of the operon. The mutational inactivation of a normally silent operon has also been described for the cryptic *E. coli bgl* operon for the utilization of β-glucosides which requires insertion of the mobile element IS5 in the promoter region to get expressed (Schnetz and Rak, 1992).

Due to its strongly increased sensitivity to several amino acids, the *B. subtilis* mutant lacking c-di-AMP is an excellent tool to study mechanisms of amino acid homeostasis, and to identify uptake and export systems. This endeavour is required as the details of amino acid transports are one of the few areas, which has several gaps of knowledge in the research on *B. subtilis* (Reuß et al., 2016). We anticipate that the further use of the c-di-AMP lacking mutant will continue to help filling these remaining gaps.

MATERIALS AND METHODS

Strains, media and growth conditions.

E. coli DH5α (Sambrook et al., 1989) was used for cloning. All *B. subtilis* strains used in this study are derivatives of the laboratory strain 168. They are listed in the Appendix. *B. subtilis* and *E. coli* were grown in Luria-Bertani (LB) or in sporulation (SP) medium (Sambrook et al., 1989; Kunst and Rapoport, 1995). For growth assays, *B. subtilis* was cultivated in MSSM medium (Gundlach et al., 2017b). MSSM is a modified SM medium in which KH_2PO_4 was replaced by NaH_2PO_4 and KCl was added as indicated (Gundlach et al., 2017b). The media were supplemented with ampicillin (100 µg/ml), kanamycin (10 µg/ml), chloramphenicol (5 µg/ml), spectinomycin (150 µg/ml), tetracycline (12.5 µg/ml) or erythromycin and lincomycin (2 and 25 µg/ml, respectively) if required.

DNA manipulation and transformation.

All commercially available restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). DNA sequences were determined by the dideoxy chain termination method (Sambrook et al., 1989). Standard procedures were used to transform *E. coli* (Sambrook et al., 1989), and transformants were selected on LB plates containing ampicillin (100 µg/ml). *B. subtilis* was transformed with plasmid or chromosomal DNA according to the two-step protocol described previously (Kunst and Rapoport, 1995). Transformants were selected on SP plates containing chloramphenicol (Cm 5 µg/ml), kanamycin (Km 10 µg/ml), spectinomycin (Spc 150 µg/ml), tetracycline (Tet 12,5 µg/ml) or erythromycin plus lincomycin (Em 2 µg/ml and Lin 25 µg/ml).

Genome sequencing.

To identify the mutations in the suppressor mutant strains GP3588, GP3638, and GP3639 (see Appendix), the genomic DNA was subjected to whole-genome sequencing. Concentration and purity of the isolated DNA was first checked with a Nanodrop ND-1000 (PeqLab Erlangen, Germany), and the precise concentration was determined using the Qubit® dsDNA HS Assay Kit as recommended by the

manufacturer (Life Technologies GmbH, Darmstadt, Germany). Illumina shotgun libraries were prepared using the Nextera XT DNA Sample Preparation Kit and subsequently sequenced on a MiSeq system with the reagent kit v3 with 600 cycles (Illumina, San Diego, CA, USA) as recommended by the manufacturer. The reads were mapped on the reference genome of *B. subtilis* 168 (GenBank accession number: NC_000964) (Barbe et al., 2009)). Mapping of the reads was performed using the Geneious software package (Biomatters Ltd., New Zealand) (Kearse et al., 2012). Frequently occurring hitchhiker mutations (Reuß et al., 2019) and silent mutations were omitted from the screen. The resulting genome sequences were compared to that of our in-house wild type strain. Single nucleotide polymorphisms were considered as significant when the total coverage depth exceeded 25 reads with a variant frequency of ≥90%. All identified mutations were verified by PCR amplification and Sanger sequencing.

Construction of mutant strains by allelic replacement.

Deletion of the *azlB*, *azlBCD*, *azlCD*, *disA*, *gltR,* and *hutH* genes was achieved by transformation of *B. subtilis* 168 with PCR product constructed using oligonucleotides to amplify DNA fragments flanking the target genes and an appropriate intervening resistance cassette as described previously (Diethmaier et al., 2014). The integrity of the regions flanking the integrated resistance cassette was verified by sequencing PCR products of about 1,100 bp amplified from chromosomal DNA of the resulting mutant strains. In the case of the *azlB, azlCD,* and *azlBCD* deletions, the cassette carrying the resistance gene lacked a transcription terminator to ensure the expression of the downstream genes.

Phenotypic analysis.

In *B. subtilis*, amylase activity was detected after growth on plates containing nutrient broth (7.5 g/l), 17 g Bacto agar/l (Difco) and 5 g hydrolyzed starch/l (Connaught). Starch degradation was detected by sublimating iodine onto the plates.

Quantitative studies of *lacZ* expression in *B. subtilis* were performed as follows: cells were grown in MSSM medium supplemented with KCl at different concentrations as indicated. Cells were harvested at OD_{600} of 0.5 to 0.8. β-Galactosidase specific activities were determined with cell extracts obtained by lysozyme treatment as described previously (Kunst and Rapoport, 1995). One unit of β-galactosidase is defined as the amount of enzyme which produces 1 nmol of o-nitrophenol per min at 28° C.

To assay growth of *B. subtilis* mutants at different histidine concentrations, a drop dilution assay was performed. Briefly, precultures in MSSM medium at the indicated histidine concentration

were washed three times, resuspended to an OD_{600} of 1.0 in MSSM basal salts solution. Dilution series were then pipetted onto MSSM plates containing the desired histidine concentration.

Plasmid constructions.

Plasmid pAC7 (Weinrauch et al., 1991) was used to construct translational fusions of the potential *yrdF* and *azlB* promoter regions to the promoterless *lacZ* gene. For this purpose, the promoter regions were amplified using oligonucleotides that attached EcoRI and BamHI restriction to the ends of the products. The fragments were cloned between the EcoRI and BamHI sites of pAC7. The resulting plasmids were pGP3807 and pGP3808 for *yrdF* and *azlB*, respectively.

To allow ectopic expression of the *azlC* and *azlCD* genes, we constructed the plasmids pGP3811 and pGP3812, respectively. The corresponding genes were amplified using oligonucleotides that added BamHI and PstI sites to the ends of the fragments and cloned into the integrative expression vector pGP1460 (Mehne et al., 2013) linearized with the same enzymes.

Northern blot analysis.

The strains *B. subtilis* 168 (wild type) and GP2222 (Δ*dac* mutant) as well as the suppressor mutants GP3638 and GP3639 were grown in MSSM minimal medium and harvested in the late logarithmic phase. The preparation of total RNA and Northern blot analysis were carried out as described previously (Schilling et al., 2007; Ludwig et al., 2002). Digoxigenin (DIG) RNA probes were obtained by *in vitro* transcription with T7 RNA polymerase (Roche Diagnostics) using PCR-generated DNA fragments as templates. The reverse primer contained a T7 RNA polymerase recognition sequence. *In vitro* RNA labelling, hybridization and signal detection were carried out according to the instructions of the manufacturer (DIG RNA labelling kit and detection chemicals; Roche Diagnostics).

Determination of intra- and extracellular histidine pools.

For the determination of histidine levels of *B. subtilis*, cells were cultivated in MSSM minimal medium until exponential growth phase (OD_{600} of 0.4). For the extraction of intracellular metabolites, 4 ml of each culture were harvested by filtration (Kohlstedt et al., 2014). Histidine levels were then determined as described previously (Guder et al., 2017) using ^{13}C labelled histidine from an *E. coli* extract as internal standard. Briefly, an Agilent 1290 Infinity II UHPLC system (Agilent Technologies) was used for liquid chromatography. The column was an Acquity BEH Amide 30 x 2.1 mm with 1.7 µm particle size (Waters GmbH). The temperature of the column oven was 30°C, and the injection volume was 3 µl. LC solvent A was: water with 10 mM ammonium formate and 0.1 % formic acid (v/v), and LC solvent B was: acetonitrile with 0.1 % formic acid (v/v). The gradient was: 0 min 90% B; 1.3 min 40 % B; 1.5 min 40 %

B; 1.7 min 90 % B; 2 min 90 % B; 2.75 min 90% B. The flow rate was 0.4 ml min^{-1}. From minute 1 to 2, the sample was injected to the MS. An Agilent 6495 triple quadrupole mass spectrometer (Agilent Technologies) was used for mass spectrometry. Source gas temperature was set to 200°C, with 14 l min^{-1} drying gas and a nebulizer pressure of 24 psi. Sheath gas temperature was set to 300°C and flow to 11 l min^{-1}. Electrospray nozzle and capillary voltages were set to 500 and 2500 V, respectively. Isotope-ratio mass spectrometry with ^{13}C internal standard was used to obtain relative data. Fully ^{12}C- and ^{13}C-labelled histidine was measured by multiple reaction monitoring in positive ionization mode using a collision energy of 13 eV. Precursor ion masses were 156 Da and 162 Da, product ion masses 110 Da and 115 Da for ^{12}C- and ^{13}C-histidine, respectively. Ratios between ^{12}C- and ^{13}C-labelled histidine were normalized to the ODs and the median ratio of the control strain 168.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG) within the Priority Program SPP1879 (Stu 214-16) (to J.S.). H.L. and T.S. acknowledge funding from the Cluster of Excellence EXC 2124 from the Deutsche Forschungsgemeinschaft. The funders had no role in study design, data collection, analysis and interpretation, decision to submit the work for publication, or preparation of the manuscript. Anja Poehlein und Rolf Daniel are acknowledged for the genome sequencing.

Author contributions: Design of the study: J.M. and J.S. Experimental work: J.M., T.S., B.M.H., and K.S. Data analysis: J.M., T.S., H.L. and J.S. Wrote the paper: J.M. and J.S.

Chapter 4 – Control of asparagine homeostasis in *Bacillus subtilis*: Identification of promiscuous amino acid importers and exporters

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Available on bioRxiv, DOI: 10.1101/2023.12.05.570048

ABSTRACT

Amino acids are the main building block for proteins. The Gram-positive model bacterium *B. subtilis* is able to import all proteinogenic amino acids from the environment as well as to synthesize them. However, the players involved in the acquisition of asparagine have not yet been identified for this bacterium. In this work, we used D-asparagine as a toxic analog of L-asparagine to identify asparagine transporters. This revealed that D- but not L-asparagine is taken up by the malate/lactate antiporter MleN. Specific strains that are sensitive to the presence of L-asparagine due to the lack of the second messenger cyclic di-AMP or due to the intracellular accumulation of this amino acid were used to isolate and characterize suppressor mutants that were resistant to the presence of otherwise growth-inhibiting concentrations of L-asparagine. These screens identified the broad-spectrum amino acid importers AimA and BcaP as responsible for the acquisition of L-asparagine. The amino acid exporter AzlCD allows detoxification of L-asparagine in addition to 4-azaleucine and histidine. This work supports the idea that amino acids are often transported by promiscuous importers and exporters. However, our work also shows that even stereo-enantiomeric amino acids do not necessarily use the same transport systems.

IMPORTANCE

Transport of amino acid is a poorly studied function in many bacteria, including the model organism *Bacillus subtilis*. The identification of transporters is hampered by the redundancy of transport systems for most amino acids as well as by the poor specificity of the transporters. Here, we apply several strategies to use the growth-inhibitive effect of many amino acids under defined conditions to isolate suppressor mutants that exhibit either reduced uptake or enhanced export of asparagine, resulting in the identification of uptake and export systems for L-asparagine. The approaches used here may be useful for the identification of transporters for other amino acids both in *B. subtilis* and other bacteria as well.

KEYWORDS

Bacillus subtilis, amino acids, asparagine metabolism, AimA, AzlB, BcaP, understudied proteins, MleN

INTRODUCTION

Every living cell is dependent on a steadily available pool of amino acids to maintain constant protein biosynthesis. The Gram-positive model organism *Bacillus subtilis* is able to synthesize all twenty proteinogenic amino acids, while using glucose and ammonium as the only sources of carbon and nitrogen, respectively. Amino acid homeostasis can be managed by the interplay of uptake, biosynthesis, degradation, export, and consumption for multiple metabolic purposes. The pathways of amino acid biosynthesis and degradation are well understood for *B. subtilis* and many other bacteria (Reuß et al., 2016). In contrast, the transport of amino acids has recently been identified as a topic that requires much more attention as there are still several amino acids, among them asparagine, glycine, phenylalanine, and tyrosine for which no transporter has been identified so far. On the other hand, no substrate has so far been identified for many potential amino acid transporters (Wicke et al., 2023). These gaps in our knowledge need to be filled to gain a comprehensive picture of amino acid homeostasis in *B. subtilis*, one of the most intensively studied organisms.

The identification of amino acid transporters is hampered by two problems: First, many amino acids are taken up by multiple transporters, and the deletion of one or even several candidate genes may not give rise to a clear phenotype. On the other hand, many amino acid transporters are promiscuous, *i.e.* they can transport multiple amino acids. Both issues have also been observed in *B. subtilis*. For example, glutamate can be transported by the amino acid permeases GltT and AimA (Klewing et al., 2020; Krüger et al., 2021). For the branched-chain amino acids isoleucine and valine, BcaP, BrnQ, and BraB have been identified as permeases (Belitsky, 2015). Similarly, three permeases, *i.e.* PutP, OpuE, and GabP, are involved in the acquisition of proline (von Blohn et al., 1997; Moses et al., 2012; Zaprasis et al., 2014). Concerning transporter promiscuity, the amino acid transporter AimA is involved in the acquisition of serine and glutamate, whereas BcaP, which was originally identified as a transporter for branched-chain amino acids, also transports threonine and serine (Klewing et al., 2020; Krüger et al., 2021; Belitsky, 2015; Commichau et al., 2015a). Similarly, the bipartite amino acid exporter AzlCD can export a branched-chain amino acid analog, 4-azaleucine as well as histidine (Belitsky et al., 1997; Meißner et al., 2022). Amino acid transporters can be identified by expressing them in established mutants that lack transporters for particular amino acids and that are therefore unable to utilize the target amino acid as a carbon and/or nitrogen source. In this way, by complementation of *Escherichia coli* mutants, AimA could be shown to transport both serine and glutamate (Klewing et al., 2020; Krüger et al., 2021). Alternatively, strains that are auxotrophic for a given amino acid can be tested for the effect of the inactivation of putative transporter gene(s). This strategy was used to identify the alanine permease AlaP (Sidiq et al., 2021). Finally, the identification of transport systems can profit from the fact that some amino acids inhibit the growth of bacteria. For threonine and serine, this growth inhibition has been described in both *E. coli* and *B. subtilis* (Lamb and

Bott, 1979b; Lamb and Bott, 1979a; Lachowicz et al., 1996; de Lorenzo et al., 2015). Some amino acids become toxic if they accumulate due to the lack of the corresponding catabolic enzymes. Similarly, strains lacking the essential second messenger c-di-AMP are highly sensitive to multiple amino acids (Krüger et al., 2021; Meißner et al., 2022; Herzberg et al., 2023), probably resulting from the formation of glutamate which drastically increases the affinity of the the potassium channel KtrCD for potassium thus supporting potassium uptake which is toxic for such mutants (Gundlach et al., 2017b; Krüger et al., 2020; Stülke and Krüger, 2020). Finally, many D-amino acids are toxic for the cells, and toxic analogs exist for many amino acids, such as 4-azaleucine (Leiman et al., 2013; Ward and Zahler, 1973). Irrespective of the context, amino acid toxicity has been proven a powerful tool to identify amino acid transporters, particularly by isolating suppressor mutants that are viable in the presence of otherwise toxic amino acids, and the identification of the responsible mutations. In this way, transporters for glutamate, proline, serine, and threonine have been discovered (Klewing et al., 2020; Krüger et al., 2021; Belitsky, 2015; Zaprasis et al., 2014; Commichau et al., 2015a).

We have a long-standing interest in amino acid metabolism and transport in *B. subtilis* (Ludwig et al., 2002; Commichau et al., 2008; Gunka et al., 2012; Gunka et al., 2013; Stannek et al., 2015; Rosenberg et al., 2016; Dormeyer et al., 2019; Warneke et al., 2023). In this work, we have addressed the transport of asparagine. Asparagine is one of the amino acids for which no importer has been identified so far in *B. subtillis*. Asparagine is synthesized from aspartate, via the asparagine synthetases AsnO, AsnH and AsnB. These enzymes all use glutamine as the amide group donor to form asparagine from aspartate. The degradation of asparagine via the asparaginases AnsA and AnsZ leads back to aspartate. Aspartate then feeds into the citric acid cycle via the aspartase AnsB. The asparaginase and aspartase are encoded in the asparagine-induced *ansAB* operon (Fisher and Wray, 2002), which also includes *mleN*, coding for a malate/lactate antiporter (Wei et al., 2000), as well as the *mleA* gene, which encodes the malic enzyme MleA that decarboxylates malate to pyruvate (Lerondel et al., 2006; Meyer and Stülke, 2013; Nicolas et al., 2012).

Using a variety of suppressor screens, we provide evidence that the malate/lactate antiporter MleN is responsible for the uptake of D-asparagine whereas AimA and BcaP are responsible for the uptake of L-asparagine. The latter amino acid can also be exported by mutational activation of the AzlCD amino acid exporter.

RESULTS

D-Asn is toxic for *B. subtilis*, and mutations in the *mleN* gene overcome this toxicity. Several D-amino acids are harmful to *B. subtilis*, as they exert toxic effects during protein synthesis (Leiman et al., 2013). We tested the effect of D-Leu, D-Arg, D-Gln, D-Asp and D-Asn on the growth of *B. subtilis*. Of these D-amino acids only D-Asn and D-Gln showed toxic effects (the results with D-Asn are shown in Fig. 10).

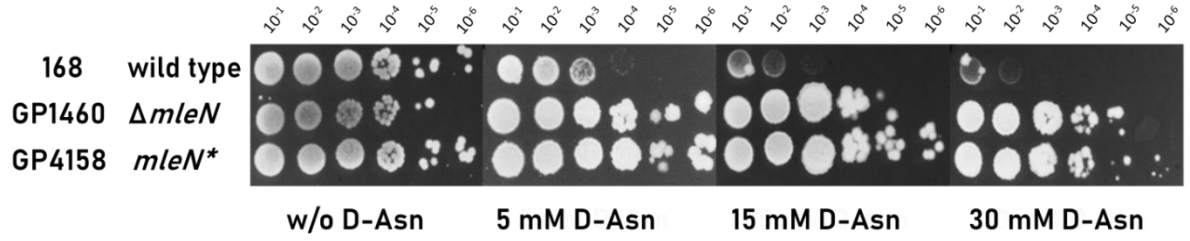

Figure 10 The *mleN* mutation confers resistance to D-asparagine stress. Sensitivity of wild type *B. subtilis* (168) and the isogenic Δ*mleN* mutant (GP1460) as well as a suppressor mutant (GP4158) isolated in the presence of D-asparagine, which carries a mutation in *mleN*. The cells were grown in SM minimal medium to an OD_{600} of 1.0 and were then diluted 10-fold to create dilutions ranging from 10^{-1} to 10^{-6}. The dilution series was dropped onto SM plates with and without D-asparagine (0, 5, 15 and 30 mM respectively). The plates were incubated at 37°C for 48 h.

The wild type strain *B. subtilis* 168 was unable to grow at D-Asn concentrations above 5 mM. We isolated stable suppressor mutants that were able to grow in the presence of D-Asn. We isolated four suppressor mutants and subjected two of them to whole genome sequencing. The data revealed an identical single mutation in the malate/lactate antiporter MleN (Wei et al., 2000) in both mutants, causing the insertion of two base pairs, resulting in a premature stop codon, and thus likely in the inactivation of the transporter (see Table 3).

Table 3 Identification of suppressor mutations[a]

Strain	Affected gene(s)	Mutation(s)
B. subtilis 168 (wild type), selection on SM plates containing D-Asn (5 mM)		
GP4158	*mleN*	+GC at position 517, frameshift, premature stop codon
GP4159	*mleN*	+GC at position 517, frameshift, premature stop codon
S3	*mleN*	+T at position 691, frameshift, premature stop codon
S4	*mleN*	+TG at pos 517, frameshift, premature stop codon
B. subtilis GP2222 (Δ*dac*), selection on MSSM plates containing L-Asn (15 mM)		
GP4269	*ktrD*	–T at position 72, frameshift, premature stop codon
GP4270	*ktrD*	–CAGTCGG at position 935, frameshift, premature stop codon
S3	*ktrD*	$ktrD_{G314W}$

S4	*ktrD*	*ktrD*$_{G314W}$
B. subtilis GP4197 (Δ*ansAB* Δ*ansZ*), selection on C glucose plates containing L-Asn		
GP4230 (5mM L-Asn)	*azlB*	*azlB*$_{N24S}$
GP4231 (10mM L-Asn)	*azlB*	+CATTAATG at position 37, frameshift, premature stop codon
GP4232 (15mM L-Asn)	*azlB*	C_{388}→T, Q130 → Stop codon, premature stop codon at pos. 130
GP4233 (30mM L-Asn)	*azlB*	*azlB*$_{D19G}$
B. subtilis GP4245 (Δ*ansAB* Δ*ansZ* Δ*azlBCD* Δ*aimA*), selection on C glucose plates containing L-Asn (isolated on disc diffusion plates, see Fig. 4)		
S1	*bcaP*	C_{982} → T, Q328 → Stop codon, premature stop codon at pos. 328
S2	*bcaP*	C_{982} → T, Q328 → Stop codon, premature stop codon at pos. 328
GP4251	*bcaP* *ydeC*	*bcaP*$_{A68E}$ *ydeC*$_{H43Q}$
S4	*bcaP*	C_{982} → T, Q328 → Stop codon, premature stop codon at pos. 328
S5	*bcaP*	–50 bp at position 153, frameshift, premature stop codon
S6	*bcaP*	–50 bp at position 153, frameshift, premature stop codon
GP4252	*bcaP*	C_{982} → T, Q328 → Stop codon, premature stop codon at pos. 328
S8	*bcaP*	BcaP$_{V92M}$
B. subtilis BP269 (SP1 Δ*ansAB*), selection on LB plates containing L-Asn (19 mM)		
BP391	*aimA* *yetL*	Duplication of 191CTA193, insertion of Thr after Ser-64 YetL$_{A96E}$
BP392	*aimA*	+G at position 213, frameshift, premature stop codon

[a] For the complete genotypes of all strains, see Appendix. All gene designations are used in *Subti*Wiki (Pedreira et al., 2022), and the genes can be retrieved there.

We then sequenced the *mleN* region of the two remaining suppressor mutants, and found that the *mleN* gene was mutated there as well. Interestingly, all mutants had insertions of one or two base pairs in the *mleN* coding region that caused frame shifts in the N-terminal half of the protein.

We then examined the growth of an *mleN* deletion strain (GP1460), compared to the wild type strain (168) and one of the suppressors (GP4158), in the presence of increasing amounts of D-Asn (see Fig. 10). While low amounts of D-Asn (up to 5 mM) could be tolerated by the cells, higher amounts led to cell death and suppressor formation in the wild type strain. Both the suppressor mutant GP4158

and the Δ*mleN* deletion mutant GP1460 were able to grow at up to 30 mM D-Asn. This result indicates that the truncation of the MleN protein in the suppressor mutant indeed results in an inactivation of the protein, and shows that this mutation is responsible for the suppression. The fact that the Δ*mleN* deletion led to full D-Asn resistance suggests that MleN can transport D-Asn in *B. subtilis*.

Expression of the *mleN* gene. The putative D-Asn transporter MleN and the NAD^+-dependent malate dehydrogenase MleA seem to be expressed in an L-asparagine-inducible operon with the asparaginase AnsA and the aspartase AnsB (Fisher and Wray, 2002; Nicolas et al., 2012). According to a large-scale transcriptome analysis with *B. subtilis* and the *Subti*Wiki database, there might be one promoter in front of *ansA* and a second promoter in front of *mleN* (Nicolas et al., 2012; Pedreira et al., 2022). We wanted to verify this observation, as well as examine a possible induction of the operon by amino acids, particularly by the D- and L-enantiomers of asparagine and aspartate. For this purpose, we made use of strains that carry fusions of the regions upstream of the *ansA* and *mleN* genes to a promoterless *lacZ* reporter gene. The strains were grown in C glucose minimal medium in the absence or presence of casein hydrolysate, and the asparagine and aspartate enantiomers (see Table 4). In agreement with previous reports (Fisher and Wray, 2002; Flórez et al., 2011), the *ansA* promoter was highly active in the presence of L-Asn, whereas only weak expression was observed under all other tested conditions. In contrast, only weak activities were observed for the *mleN* promoter. The observed readthrough in the *ansAB-mleNA* region (Nicolas et al., 2012) as well as the absence of a promoter in front of *mleN* suggest, that the four genes are co-expressed in the *ansAB-mleNA* operon, via the asparagine-inducible promoter in front of *ansA*. Due to the toxicity of D-Asn, we were unable to assay promoter activities in the wild type background. To circumvent this problem, we transferred the *lacZ* fusion constructs into the strain lacking the D-asparagine importer MleN. In this case (GP4191), we observed a very weak increase of *ansA* promoter actvity which is, however, neglegible as compared to the observed induction of the promoter in the presence of L-Asn (Table 4) and in comparison to promoters of basic carbon metabolism (Schilling et al., 2007). However, this activity seems to be sufficient to express the *mleN* gene to a level that allows uptake of D-Asn and concomitant intoxication of the *B. subtilis* cells.

Table 4. Activity of the putative *mleN* and *ansA* promoters.

		Units of β-galactosidase per µg of protein Addition to C-Glc minimal medium					
Strain	**Relevant genotype**	**None**	**CAA**	**D-Asn**[a]	**L-Asn**[a]	**D-Asp**[a]	**L-Asp**[a]
GP829	*mleN-lacZ*	6 ± 1	11 ± 7	NG[b]	2 ± 0	56 ± 15	2 ± 0
GP1155	*ansA-lacZ*	13 ± 1	52 ± 3	NG	1325 ± 247	18 ± 2	11 ± 2
GP4191	*ansA-lacZ* Δ*mleN*	11 ± 2	47 ± 5	48 ± 23	1798 ± 83	30 ± 4	24 ± 7

[a] The amino acids were added to a concentration of 5 mM; [b] NG, no growth.

The toxicity of L-Asn for a Δ*dac* mutant allows the identification of AimA as an L-Asn transporter. In some cases, the D- and L-enantiomers of an amino acids are taken up by the same transporter, as it is the case for D- and L-alanine that are both taken up by AlaP (Sidiq et al., 2021). This raised the question whether MleN might also be involved in the transport of L-Asn. This hypothesis is highly attractive since *mleN* is part of an operon involved in L-Asn utilization that is induced by this amino acid. The *B. subtilis* wild type strain 168 tolerates L-Asn in minimal medium (see Fig. 11).

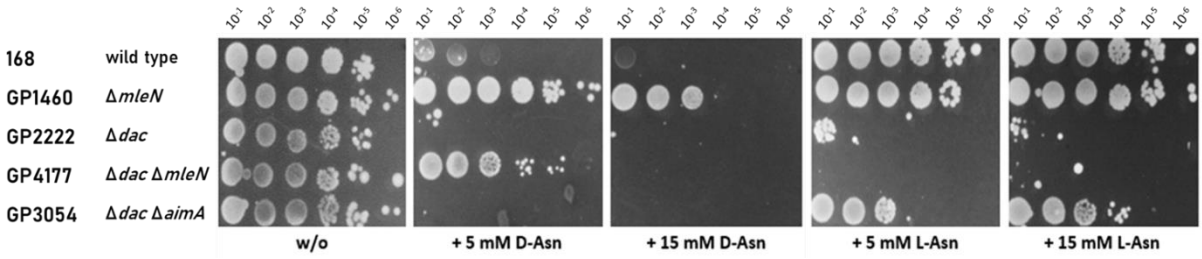

Figure 11 MleN is specific to D-asparagine import, while AimA is an L-asparagine importer. Sensitivity of wild type *B. subtilis* (168), the Δ*mleN* mutant (GP1460) and Δ*dac* mutants to D-Asn and L-Asn is compared on MSSM minimal medium. The cells were grown in MSSM minimal medium to an OD_{600} of 1.0 and were then diluted 10-fold to create dilutions ranging from 10^{-1} to 10^{-6}. The dilution series was dropped onto MSSM plates with and without L-asparagine. The plates were incubated at 42°C for 48 h.

We made therefore use of the observation that a strain that is unable to produce c-di-AMP due to the deletion of the three genes encoding the diadenylate cyclases (Δ*dac*) is sensitive to glutamate and several other amino acids (Krüger et al., 2021; Herzberg et al., 2023). As shown in Fig. 11, the Δ*dac* mutant GP2222 is highly sensitive to growth inhibition by both D-Asn and L-Asn. To test the role of MleN in the uptake of L-Asn, we constructed the Δ*dac* Δ*mleN* mutant GP4177. Previous studies have shown that AimA is a major low affinity glutamate and serine importer in *B. subtilis* (Klewing et al., 2020; Krüger et al., 2021). Thus, we also used the Δ*dac* Δ*aimA* mutant strain GP3054 (Fig. 11). The wild

type as well as the single and double mutants were tested for growth in the absence and presence of D- and L-Asn. All strains grew well in the absence of any added amino acid. As expected, growth of the wild type strain 168 and the Δ*dac* mutant was inhibited In the presence of D-Asn whereas the deletion of *mleN* restored growth in both genetic backgrounds. In addition, the Δ*dac* Δ*aimA* double mutant was unable to grow in the presence of D-Asn, confirming that AimA does not contribute to the uptake of D-Asn. L-Asn was well tolerated by both the wild type and the Δ*mleN* mutant. In contrast, the Δ*dac* mutant GP2222 was unable to grow if L-Asn was present. The analysis of the double mutants revealed that only the deletion of *aimA* but not of *mleN* confers resistance to L-Asn. The drop dilution assay (see Fig. 11) shows that the deletion of *aimA* allows growth in the presence of L-Asn; however, growth was not restored to the full level as compared to the wild type strain, indicating that *B. subtilis* encodes additional L-Asn transporter(s). Taken together, our results indicate that MleN does not contribute to the uptake of L-asparagine, whereas AimA is able to transport this amino acid. This experiment demonstrates that the L- and D-forms of Asn, though structurally very similar, are not taken up by the same transporter.

To get more insights into the sensitivity of the Δ*dac* mutant GP2222 to L-asparagine, we isolated suppressor mutants that were able to grow in the presence of 15 mM L-asparagine. Of four isolated mutants, two were subjected to whole-genome sequencing. Both mutants had frameshift mutations in the *ktrD* gene that result in the formation of truncated proteins (see Table 3). The *ktrD* alleles of the two remaining mutants were sequenced, and both had nucleotide substitutions that resulted in the replacement of Gly-314 by a Trp residue. The KtrD protein is the membrane subunit of the low affinity KtrCD potassium channel (Holtmann et al., 2003). It has been shown that the presence of glutamate results in high-affinity potassium uptake by KtrCD, resulting in intoxication of the Δ*dac* mutant (Krüger et al., 2020). It is therefore possible that either L-asparagine itself can also activate KtrCD, or that KtrCD is activated by the aspartate formed upon asparagine degradation. The possible link between KtrCD and asparagine transport will be addressed elsewhere. The fact that we did not find suppressor mutations with inactivated amino acid transporter genes supports the idea that multiple transporters for L-Asn are present in *B. subtilis*.

Identification of the AzlCD system as an potential exporter for L-Asn. Amino acids can be growth-inhibiting by themselves or due to the generation of harmful intermediates (de Lorenzo et al., 2015). To distinguish between these possibilities, we investigated the toxicity of L-asparagine in a strain that lacks the two asparaginases AnsA and AnsZ. This strain (GP4197) is sensitive to L-asparagine on minimal medium suggesting that the accumulation of L-asparagine itself is harmful for the cells (see Fig. 12). We observed suppressor mutant formation of the *ΔansAB ΔansZ* strain GP4197 after incubation at 42°C for 48h with 5, 10, 15 and 30 mM L-asparagine. We isolated four suppressor mutants, one for each of the tested concentrations of L-asparagine.

To identify the mutations responsible for the resistance to L-Asn, we performed whole genome sequencing for the four suppressor mutants. The sequencing results are summarized in Table 3. In each mutant, we found single mutations that all affected the transcriptional repressor AzlB, which controls the expression of the *azl* operon. Two of the mutations (in GP4231 and GP4232) result in the formation of truncated proteins (due to a frame shift or a point mutation that converts a codon for glutamine to a stop codon), and two mutations (in GP4230 and GP4233) result in AzlB proteins with amino acid substitutions. Two of the mutations, the eight base pair insertion of CATTAATG after nucleotide 37 (in GP4231) and the Asn24-Ser substitution (in GP4230) were already found in a previous study as a result of selection for resistance to histidine of the Δ*dac* strain (Meißner et al., 2022). It is therefore likely, that the *azlB* gene is inactivated in all suppressor mutants. The inactivation of *azlB* causes overexpression of the genes of the *azl* operon that are expressed downstream of *azlB* (Belitsky et al., 1997; Meißner et al., 2022). This involves the bipartite amino acid exporter AzlCD. To test the role of AzlB and AzlCD in the resistance to and export of L-Asn, we constructed strains carrying deletions of *azlB* and *azlBCD* and performed drop dilution assays with the resulting Δ*ansAB* Δ*ansZ* Δ*azlB* and Δ*ansAB* Δ*ansZ* Δ*azlBCD* triple mutants (GP4236 and GP4237, respectively) (Fig. 12). We observed improved growth when *azlB* was mutated or deleted, demonstrating that the inactivation of the *azlB* gene is responsible for the resistance to L-Asn. The additional deletion of the *azlCD* genes encoding the amino acid exporter resulted in the loss of growth. This finding suggests that AzlCD exports L-Asn in addition to 4-azaleucine and histidine (Belitsky et al., 1997; Meißner et al., 2022) (see Fig. 16).

AimA is the major transporter for L-Asn on complex medium. The *B. subtilis* strain SP1 is a prototrophic derivative of the laboratory strain 168 that has been used throughout this study. Due to its prototrophy this strain is of interest for biotechnological applications (Richts et al., 2020). SP1 lacking the *ansAB* operon (strain BP269) is also sensitive to L-Asn, even on complex medium. Again, we were able to isolate suppressor mutants that could tolerate the addition of 19 mM L-Asn to LB medium. Genome sequencing of two suppressor mutants (see Table 3) revealed two distinct mutations that affect the amino acid importer AimA. In the mutant BP391, there was an insertion of three bases in the *aimA* gene resulting in the insertion of a Thr residue after Ser-64 in AimA. In the second mutant, BP392, there was a single nucleotide insertion in *aimA*, resulting in the expression of a truncated protein. In addition, BP391 had a second mutation that resulted in the substitution of Ala-96 by a Glu residue in the YetL protein, which acts as a transcriptional repressor to control the expression of the *yetM* (encodes a FAD-dependent monooxygenase) and *yetL* genes (Hirooka et al., 2009). Since these functions seem to be unrelated and the inactivation of AimA was already shown to be sufficient to cause resistance to L-Asn, the *yetL* mutation was not further investigated. In conclusion, the suppressor screen using the SP1-derived strain BP269 confirms the important role of AimA in the uptake of L-Asn.

As we have identified AimA as an importer for L-asparagine, we also deleted the *aimA* gene in the Δ*ansAB* Δ*ansZ* mutant in order to test whether the strain would still be sensitive to L-Asn stress on minimal medium. Interestingly, the deletion of the *aimA* gene only conferred limited resistance to L-asparagine (see Fig. 12, GP4239) if the bacteria grew on minimal medium. Again, this finding indicates that there are multiple transporters for L-Asn in *B. subtilis*.

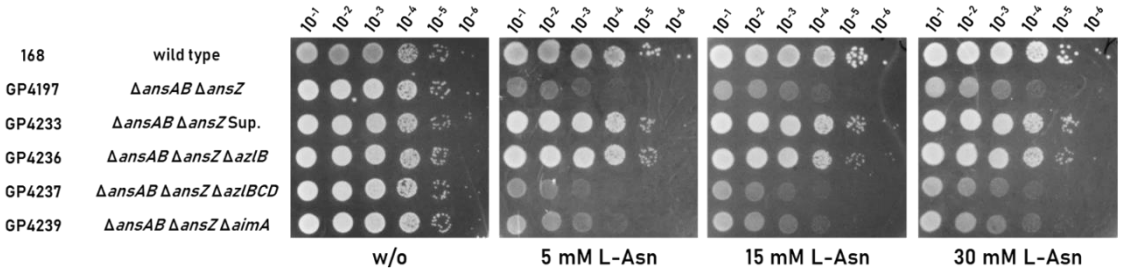

Figure 12 AzlCD provides resistance to L-asparagine when AzlB is mutated. Sensitivity of the Δ*ansAB* Δ*ansZ* (GP4197) double mutant is shown compared to the suppressor GP4233, isolated from L-Asn stress, as well as the knockout strains for the exporter AzlCD and the importer AimA. The cells were grown in C-Glc minimal medium to an OD_{600} of 1.0 and were then diluted 10-fold to create dilutions ranging from 10^{-1} to 10^{-6}. The dilution series was dropped onto C-Glc plates with and without L-asparagine (0, 5, 15 and 30 mM respectively). The plates were incubated at 42°C for 48 h.

Further adaptation to L-Asn identifies BcaP as additional L-Asn importer. In order to increase the selective pressure, we employed a disc diffusion assay of a strain that lacks the main known options to adapt to the presence of L-Asn (the Δ*ansAB* Δ*ansZ* Δ*azlBCD* Δ*aimA* quadruple mutant GP4245) with high concentrations of L-Asn (500 mM) (see Fig. 13). We observed suppressor mutant formation after 48h of incubation at 42°C. We isolated a total of eight suppressors and sequenced the whole genomes of two of them. The results are summarized in Table 3. Both mutants carried mutations affecting the amino acid transporter BcaP. In strain GP4251, BcaP had a substitution of Ala-68 in Glu, and in GP4252, there was a base substitution that resulted in the direct formation of a stop codon and thus to a truncated BcaP protein. In addition, strain GP4251 had a second mutation in the *ydeC* gene that resulted in a substitution of His-43 to Gln in the AraC-type transcription factor YdeC of unknown function. We then sequenced the *bcaP* and *ydeC* alleles of the remaining six mutants. All had mutations in *bcaP*, while none of them had the mutation in *ydeC*. Therefore, we focussed the further analysis on *bcaP*.

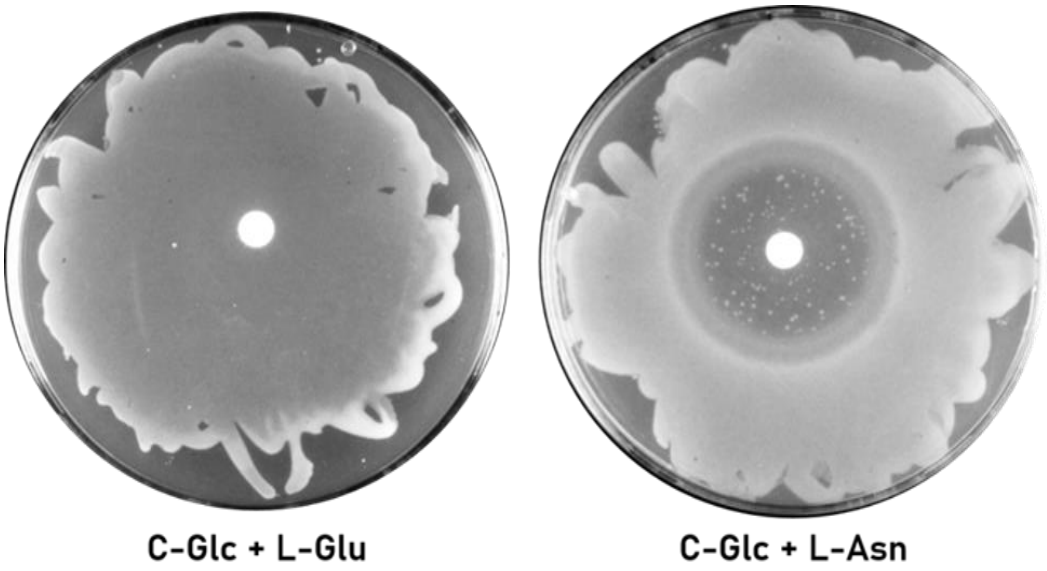

Figure 13 Disc diffusion assay of the Δ*ansA* Δ*ansZ* Δ*azlBCD* Δ*aimA* (GP4245) strain. 15 µl 150 mM L-glutamate solution was used as positive control and dropped on the filter disc. 15 µl 500 mM L-asparagine solution was used to apply the selective pressure. Suppressor mutants appeared after 48h of incubation at 42°C.

All eight suppressors carried mutations in *bcaP*, the gene coding for the branched chain amino acid permease BcaP, which is involved in the uptake of isoleucine, valine, threonine and serine (Klewing et al., 2020; Belitsky, 2015; Commichau et al., 2015a). The mutations appear to make BcaP nonfunctional, which suggests a role of BcaP in the uptake of L-asparagine. When we compared our suppressors as well as the effect of a deletion of the *bcaP* gene (in strain GP4267) with the original strain (Δ*ansAB* Δ*ansZ* Δ*azlBCD* Δ*aimA*) in a drop dilution assay, we found that both the suppressor mutants as well as the *bcaP* deletion mutant are fully resistant to L-Asn stress (see Fig. 14). Thus, the lack of both AimA and BcaP confers full resistance to L-Asn, indicating that these two transporters are responsible for the uptake of this amino acid.

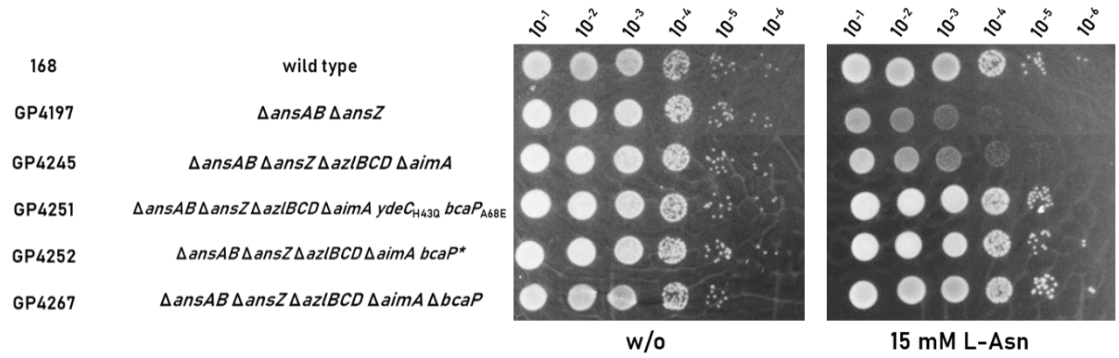

Figure 14 BcaP is also an Importer for L-Asn. Sensitivity of both suppressors (GP4251, GP4252) isolated via disc diffusion assay, as well as the Δ*ansAB* Δ*ansZ* Δ*azlBCD* Δ*aimA* Δ*bcaP* mutant (GP4267) to L-Asn is shown. The cells were grown in C-Glc minimal medium to an OD_{600} of 1.0 and were then diluted 10-fold to create dilutions ranging from 10^{-1} to 10^{-6}. The dilution series was dropped onto C-Glc plates with and without L-asparagine (0 and 15mM) respectively. The plates were incubated at 42°C for 48 h.

The AimA amino acid importer and the AzlCD amino acid exporter have been shown to be involved in the uptake and export, respectively, of multiple amino acids. In order to characterize the suppressor mutants further, we also carried out an experiment with L-serine. L-Ser is toxic to *B. subtilis* in minimal medium (Klewing et al., 2020; Lachowicz et al., 1996) To test, whether AimA, BcaP, and AzlCD are also involved in the homeostasis of L-Ser, we examined the growth of the wild type strain 168, the Δ*azlB* mutant GP3600, the Δ*ansAB* Δ*ansZ* Δ*azlBCD* Δ*aimA* mutant GP4245 and of the two suppressor mutants GP4251 and GP4252 at different concentrations of L-Ser (see Fig. 15). As observed before (Klewing et al., 2020), the wild type strain was unable to grow if L-Ser was present. The same result was obtained for the *azlB* mutant GP3600 indicating that the AzlCD amino acid exporter does not play a significant role in serine export. At L-Ser of 5 mM, the loss of the amino acid importer AimA in GP4245 provided a very faint protection against growth inhibition by L-Ser. However, both suppressor mutants that are otherwise isogenic to the progenitor GP4245, exhibited a strong resistance to the presence of serine. This oberservation is in good agreement with previous reports that BcaP and AimA make the major contributions to L-Ser uptake. Interestingly, the two suppressor mutants differ in their resistance to L-Ser at higher concentrations. The *aimA bcaP* double mutant GP4252 barely grew at 15 mM serine or above. In contrast, GP4251 which has an additional mutation affecting the AraC family regulator YdeC grew well at 15 mM of L-Ser indicating that the mutation in *ydeC* contributes to amino acid resistance (see Discussion).

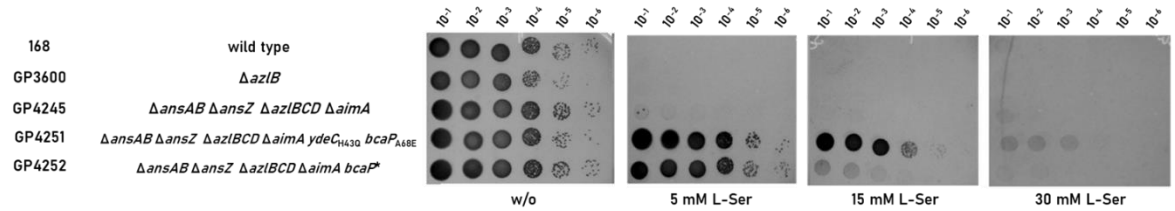

Figure 15 The isolated suppressors are also resistant against L-serine. Sensitivity of wild type *B. subtilis* (168), the Δ*azlB* mutant (GP3600), the Δ*ansAB* Δ*ansZ* Δ*azlBCD* Δ*aimA* mutant (GP4245) and the two suppressors isolated from L-Asn stress (GP4251 and GP4252) to L-serine is shown. The cells were grown in C-Glc minimal medium to an OD_{600} of 1.0 and were then diluted 10-fold to create dilutions ranging from 10^{-1} to 10^{-6}. The dilution series was dropped onto C-Glc plates with and without L-serine (0, 5, 15 and 30 mM respectively) The plates were incubated at 42°C for 48 h.

DISCUSSION

This work aimed to utilize the toxic effects of D-asparagine to identify uptake mechanisms for both D- and L-asparagine, following the hypothesis that both enantiomers enter the cell via the same transporter, as it is the case for alanine in *B. subtilis* (Sidiq et al., 2021). The mutations in MleN revealed it to be a D-Asn importer. Even in the presence of MleN, L-Asn was well tolerated if the two transporters AimA and BcaP were missing indicating that MleN is not involved in the uptake of L-Asn.

This demonstrates that, although structurally similar, D- and L-asparagine are taken up by different uptake systems. Our suppressor screen didn't give any indication of the possiblity of D-Asn export in *B. subtilis*. This is interesting since we were easily able to find mutations in the *azlB* repressor gene that result in consitutive expression of the amino acid exporter AzlCD in the presence of L-Asn. This observation again supports the idea, that even though amino acid transporters are often quite promiscous (Wicke et al., 2023), it can not be taken for granted that transporters that import or export any given amino acid do so for the L- and D-forms of the amino acid. It should be noted that the high concentration of D-Asn used in the suppressor screen does not reflect physiological conditions. Therefore, it cannot be ruled out that *B. subtilis* encodes additional high-affinity transporters for D-Asn that might be active at lower concentrations.

A critical point for the identification of bacterial amino acid transporters is the use of strains and/or conditions that make the target amino acid toxic for the cells. In this study, we used strains lacking the second messenger c-di-AMP (Δ*dac*) and strains that are unable to degrade asparagine (Δ*ansAB* mutant) resulting in the intracellular accumulation of L-Asn. Interestingly, the different strains seem to have experienced different selective pressures resulting in the identification of highly specific suppressor mutations according to the selection scheme. For the Δ*dac* mutant, the well-established sensitivity to potassium was the major bottleneck, resulting in the isolation of *ktrD* mutants in all four cases (see below). For the strain lacking both asparaginases, the intracellular accumulation of L-Asn was the main issue which could be efficiently solved by activating the AzlCD amino acid exporter in all four independently isolated strains. Interestingly, the prototrophic strain SP1 lacking the asparaginase AnsA inactivated the AimA amino acid importer rather than activating AzlCD. This might result from the use of complex medium in which several amino acids share transporters. Moreover, the expression of the second transporter BcaP is repressed during growth on complex medium by the transcription factor CodY (Molle et al., 2003). Thus, under these conditions, AimA might be the only relevant transporter for L-Asn, resulting in its preferred inactivation to cope with L-Asn stress. Finally, when degradation or export of L-Asn were blocked, and one of the uptake systems (AimA) was also missing, mutations in a second transporter BcaP were selected in all eight cases. Thus, our results highlight the specificity of the outcomes that result from even subtle differences in the selective scenario.

In the case of the Δ*dac* mutant GP2222, all suppressor mutants had the KtrD potassium channel inactivated. This protein imports potassium with low affinity, but it has a very high affinity for the ion in the presence of glutamate (Krüger et al., 2021). It can therefore be concluded that the well-established potassium sensitivity of the Δ*dac* mutant (Krüger et al., 2021; Gundlach et al., 2017b; Gundlach et al., 2019) is the major factor that causes growth inhibition in the presence of L-Asn, resulting in mutations that inactivate the strongly expressed KtrD potassium channel. Similarly, a mutation in *ktrD* was also selected if the Δ*dac* mutant grew in the presence of high concentrations of

histidine (Meißner et al., 2022). While glutamate is one one the final degradation products of histidine, no glutamate is formed during the utilization of L-Asn. This suggests that either L-Asn or its degradation product L-Asp can also cause an increased affinity of KtrD for potassium, as shown for glutamate (Krüger et al., 2021).

Our previous work has shown that the AimA protein is a major non-specific player in amino acid uptake (Klewing et al., 2020; Krüger et al., 2021). Based on our results with both the Δ*dac* Δ*aimA* mutant GP3054 (see Fig. 11) and the SP1 Δ*ansAB* mutant BP269 (see Table 3), AimA also plays the major role in the uptake of L-Asn. Thus, AimA is the major importer for glutamate, serine and also asparagine (Klewing et al., 2020; Krüger et al., 2021). It is tempting to speculate that AimA might also be involved in the transport of other amino acids. Since AimA was discovered only recently, its presence may have masked previous genetic attempts to identify amino acid transporters in *B. subtilis*. AimA is a member of the large amino acid-polyamine-organocation superfamily (Reizer et al., 1993). These proteins typically transport amino acids, but they may also be involved in the uptake of methyl-thioribose (MtrA) or potassium ions (KimA) (Gundlach et al., 2017b; Borriss et al., 2018). Interestingly, AimA seems to be limited to *B. subtilis*, as close orthologs of the protein are missing in most species, even in other *Bacillus* species. In *B. subtilis*, AimA has a paralog, YveA. This protein is expressed during sporulation in the forespore (Wang et al., 2006) and may thus be important for amino acid uptake once the spores start to germinate. However, based on the expression profile, YveA is unlikely to contribute to amino acid transport in growing cell.

Based on the suppressor mutants, the selective pressure caused by L-Asn was different in strains that are unable to degrade this amino acid from those that lack c-di-AMP. In the case of the 168-derived *ansAB ansZ* mutant all mutations affected the AzlB transcription repressor. Such mutations were previously shown to cause expression of the amino acid exporter complex AzlCD and to allow export of toxic histidine (Meißner et al., 2022). Moreover, an amino acid export activity of AzlCD was also suggested for 4-azaleucine (Belitsky et al., 1997). Indeed, the loss of the exporter subunits in addition to the repressor results in an increased sensitivity towards L-Asn as compared to the *azlB* repressor mutant (see Fig. 14). This suggests that the AzlCD amino acid exporter is responsible for the resistance to L-Asn by exporting this amino acid. However, we cannot exclude the possibility that the accumulation of L-Asn in the *ansAB ansZ* mutant results in the formation of toxic products that are the actual substrate for export by AzlCD. Taking into account that AzlCD is a member of a family of amino acid exporters (Eggeling and Sahm, 2003), and all the observations of this study it seems most likely that L-Asn is indeed the substrate for AzlCD.It is interesting to note that L-Asn is already the third amino acid that is likely to be exported by the AzlCD exporter. Moreover, expression of AzlCD also provides resistance to otherwise toxic diaminopropionic acid (Warneke et al.,, unpublished results), thus suggesting that this complex is a broad-spectrum amino acid exporter. So

far, no conditions have been identified that would allow a substantial expression of the AzlCD amino acid exporter (Nicolas et al., 2012), and the acquisition of resistance to growth-inhibiting amino acids always depends on the inactivation of the *azlB* repressor gene. It is tempting to speculate that the untimely expression of the amino acid exporter might cause a loss of amino acids from the cell. Since the bacteria invest a lot of energy to either synthesize or import amino acids, their loss would be disadvantage for the bacteria. Indeed, the fitness of the *azlB* mutant is reduced in minimal media with ammonium as the single source of nitrogen, when the cells depend on de novo biosynthesis of amino acids (Koo et al., 2017). Thus, it might be a good strategy to encode the exporter in the genome but to keep the gene silent unless expression is required because a toxic amino acid causes the corresponding selective pressure.

As stated above, the identification of conditions that render an amino acid toxic is crucial to find the corresponding transport systems. Another step in this strategy is to use mutants that are unable to activate *e.g.* amino acid exporters or that are already defective in main transporters. Indeed, this approach allowed us to identify BcaP as a second importer for L-Asn. As for AimA, several substrates have been identified for BcaP. BcaP is the major transporter for the branched-chain amino acids isoleucine and valine as well as for threonine (Klewing et al., 2020; Belitsky, 2015; Commichau et al., 2015a). Moreover, in addition to AimA, BsaP plays a minor role in serine uptake (Klewing et al., 2020). The observation that BcaP is also involved in the acquisition of L-Asn suggests that it is also a more non-specific amino acid importer.

In one of the suppressor mutants that had acquired the mutations in *bcaP*, we found a second mutation in the *ydeC* gene encoding an unknown transcription regulator of the AraC family. It is tempting to speculate that YdeC controls the expression of an amino acid transporter. The observation that the presence of the *ydeC* mutation causes an increased resistance towards serine suggests again a general role for the corresponding transporter. Indeed, this transporter, AexA (previously YdeD), can export β-alanine (Warneke et al.,, unpublished results) and probably also L-Asn and L-Ser.

The work described here has identified systems for the uptake and export of D- and L-Asn in *B. subtilis* (see Fig. 16). As for many other proteinogenic amino acids, L-Asn can be imported and exported by multiple broad-range transport proteins.

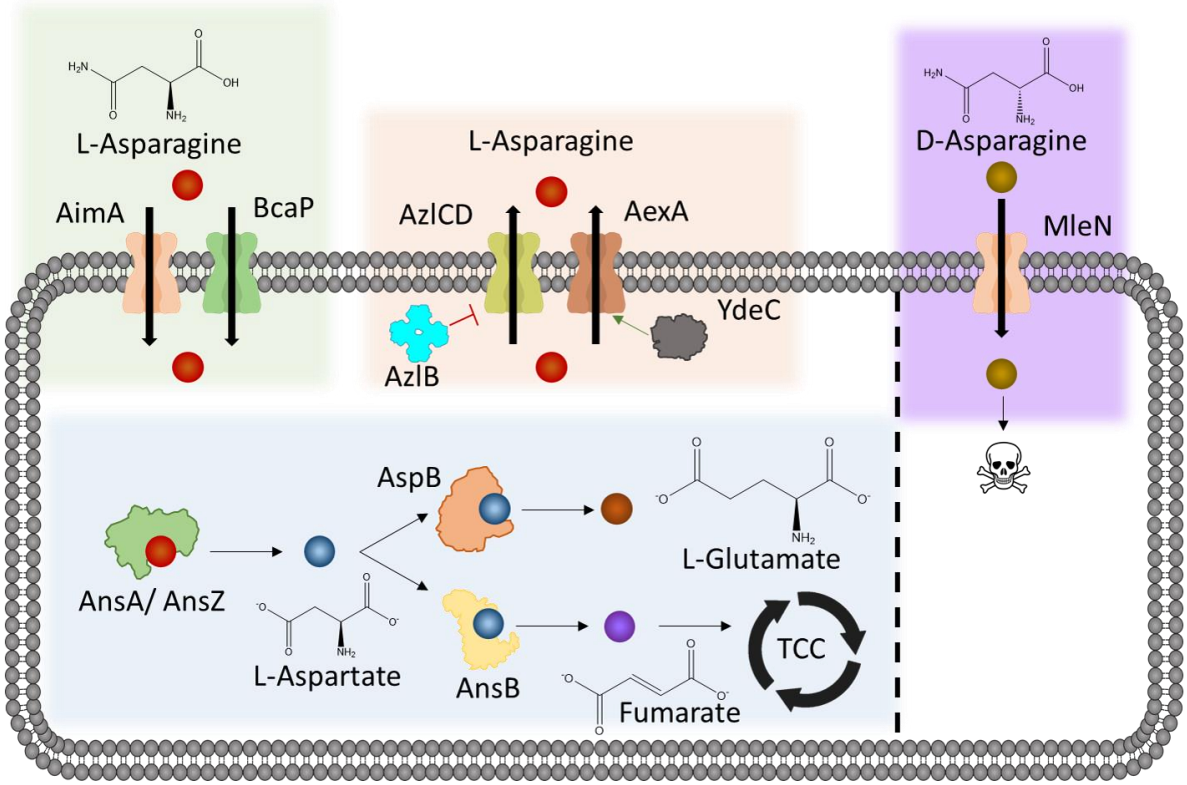

Figure 16 Asparagine metabolism in *B. subtilis*. L-asparagine enters the cell via the importers AimA and BcaP. Within the cell it is degraded to L-aspartate and then subsequently feeds into the tricarboxylic acid cycle (TCC) and production of L-glutamate via a newly discovered AspB-dependent bypass (Mardoukhi et al., 2023). L-asparagine is exported from the cell due to mutations in the regulators AzlB and YdeC. Export happens by the broad range amino acid exporters AexA (YdeD) and AzlCD. D-asparagine enters the cell via MleN and is toxic to *B. subtilis*. No form of degradation or export was detected.

MATERIALS AND METHODS

Strains, media and growth conditions. *E. coli* DH5α was used for cloning. All *B. subtilis* strains used in this study are derivatives of the laboratory strains 168 or SP1. *B. subtilis* and *E. coli* were grown in Luria-Bertani (LB) or in sporulation (SP) medium (Sambrook et al., 1989; Kunst and Rapoport, 1995). For growth assays and the *in vivo* interaction experiments, *B. subtilis* was cultivated in LB, SM, MSSM or C-Glc minimal medium (Gundlach et al., 2017b). SM is a minimal medium that uses KH_2PO_4 as buffer. MSSM is a modified SM medium in which KH_2PO_4 was replaced by NaH_2PO_4 and KCl was added as indicated. C-Glc is a chemically defined medium that contains glucose (1 g/l) as carbon source (51). The media were supplemented with ampicillin (100 µg/ml), kanamycin (10 µg/ml), chloramphenicol (5 µg/ml), spectinomycin (150 µg/ml), tetracycline (12.5 µg/ml) or erythromycin plus lincomycin (2 and 25 µg/ml, respectively) if required.

DNA manipulation and transformation. Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (Sambrook et al., 1989). All commercially available plasmids, restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. *B. subtilis* was transformed with plasmids, genomic DNA or PCR

products according to the two-step protocol (Kunst and Rapoport, 1995). DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). DNA sequences were determined by the dideoxy chain termination method (Sambrook et al., 1989).

Construction of mutant strains by allelic replacement. Deletion of the *ansAB, aimA* and *bcaP* genes was achieved by transformation of *B. subtilis* 168 with a PCR product constructed using oligonucleotides to amplify DNA fragments flanking the target genes and an appropriate intervening resistance cassette as described previously (Diethmaier et al., 2014). The integrity of the regions flanking the integrated resistance cassette was verified by sequencing PCR products of about 1,100 bp amplified from chromosomal DNA of the resulting mutant strains. In the case of *ansAB*, the cassette carrying the resistance gene lacked a transcription terminator to ensure the expression of the downstream genes.

Phenotypic analysis. In *B. subtilis*, amylase activity was detected after growth on plates containing nutrient broth (7.5 g/l), 17 g Bacto agar/l (Difco) and 5 g hydrolyzed starch/l (Connaught). Starch degradation was detected by sublimating iodine onto the plates.

Quantitative studies of *lacZ* expression in *B. subtilis* were performed as follows: cells were grown in C-Glc medium. Cells were harvested at OD_{600} of 0.5 to 0.8. β-Galactosidase specific activities were determined with cell extracts obtained by lysozyme treatment as described previously (Kunst and Rapoport, 1995). One unit of β-galactosidase is defined as the amount of enzyme which produces 1 nmol of o-nitrophenol per min at 28° C. All β-galactosidase assays were performed in triplicate.

To assay the growth of *B. subtilis* mutants at different asparagine concentrations, multiple drop dilution assays was performed. Briefly, precultures in either MSSM, C-Glc or SM minimal medium at the indicated L- and D-asparagine concentration were washed three times and resuspended to an OD_{600} of 1.0 in a 1 x MSSM buffer, C-salts or SM-buffer solution. A dilution series was then pipetted onto the respective minimal medium plates containing the desired asparagine concentration. All drop dilution assays were performed in triplicate.

Disc Diffusion Assay. For the disc diffusion assay a preculture of GP4245 in C-Glc medium was prepared. The cells were harvested during exponential growth phase, washed three times and then resuspended to an OD_{600} of 1.0 in a 1 x C-salts solution. Then, 150 µl of the cells were spread onto C-Glc agar plates and left for 2 minutes to dry. A sterile filter disc was placed in the middle of the agar plates and 15µl of highly concentrated (500 mM) L-asparagine solution was then pipetted onto the filter disc. A plate with 150 mM L-glutamate solution was used as positive control. The plates were incubated at 42°C for 48 h and then photographed and the suppressors isolated.

Genome sequencing. To identify the mutations in the suppressor mutant strains BP391, BP392, GP4158, GP4159, GP4230, GP4231, GP4232, GP4233, GP4251, GP4252, GP4269, GP4270 (see Table 3, Appendix), the genomic DNA was subjected to whole-genome sequencing. Concentration and

purity of the isolated DNA was first checked with a Nanodrop ND-1000 (PeqLab Erlangen, Germany), and the precise concentration was determined using the Qubit® dsDNA HS Assay Kit as recommended by the manufacturer (Life Technologies GmbH, Darmstadt, Germany). Illumina shotgun libraries were prepared using the Nextera XT DNA Sample Preparation Kit and subsequently sequenced on a MiSeq system with the reagent kit v3 with 600 cycles (Illumina, San Diego, CA, USA) as recommended by the manufacturer. The reads were mapped on the reference genome of *B. subtilis* 168 (GenBank accession number: NC_000964) (Barbe et al., 2009). Mapping of the reads was performed using the Geneious software package (Biomatters Ltd., New Zealand) (Kearse et al., 2012). Frequently occurring hitchhiker mutations (Reuß et al., 2019) and silent mutations were omitted from the screen. The resulting genome sequences were compared to that of our in-house wild type strain. Single nucleotide polymorphisms were considered as significant when the total coverage depth exceeded 25 reads with a variant frequency of ≥90%. All identified mutations were verified by PCR amplification and Sanger sequencing.

Plasmid construction. To construct translational fusions of the potential *mleN* and *ansA* promoter regions to the promoterless *lacZ* gene, we used the plasmids pAC7 (Weinrauch et al., 1991) and pAC5 (Martin-Verstraete et al., 1992), respectively. Briefly, the promoter regions were amplified using oligonucleotides that attached EcoRI and BamHI restriction to the ends of the products, and the fragments were cloned between the EcoRI and BamHI sites of pAC5 or pAC7. The resulting plasmids were pGP388 for *mleN* and pGP872 for *ansA*, respectively.

ACKNOWLEDGEMENTS

We are grateful to Jens Landmann, Paul Molis, and Aneta Zelazo for the help with some experiments. This work was supported by grants from the Deutsche Forschungsgemeinschaft via Priority Programme SPP1879 (to J.S. and F.M.C.).

Author contributions: Design of the study: J.M. and J.S. Experimental work: J.M., M.K. Data analysis: J.M., R.W., and J.S. *lacZ* fusion of the *ansA*-promoter region: K.W. SP1 mutants from L-Asn pressure: M. S. Y. M. and F.M.C. Wrote the paper: J.M. and J.S.

Chapter 5 – Discussion

5.1. Reasons for amino acid toxicity

B. subtilis is a soil bacterium that inhabits a broad range of biotopes. The natural biosphere ususally provides a multitude of nutrients, but also stressors that microorganisms are exposed to. *B. subtilis* is well adapted to survive in many environments, so it is ubiquitous in the natural biosphere (Earl et al., 2008). A natural soil sample contains a lot of amino acids, with glutamate, glutamine, aspartate, asparagine, alanine and histidine accounting for approximately 80% of the total amino acid pool (Werdin-Pfisterer et al., 2009; Senwo and Tabatabai, 1998). This natural richness of nutrients likely promoted the formation of amino acid transporters, as amino acids are preferably taken up rather than being synthesized within the cell. The ability to synthesize all amino acids is also not shared between all prokaryotes, as the lifestyle of certain bacteria completely functions without anabolic reactions. The pathogen *Mycoplasma pneumoniae* is a well-suited example for this development, as it relies on a parasitic lifestyle, taking up amino acids completely from its host (Himmelreich et al., 1996). By now, bacteria of the genus *Mycoplasma* are established as model organisms for bacteria with minimal genomes (Fraser et al., 1995; Himmelreich et al., 1996). This even allowed the creation of the synthetic organism; JCV-syn1.0, which is based on the genome of *M. mycoides* (Gibson et al., 2010). Further deletions left this organism with 31 genes involved in membrane transport, although the substrates for each of the transporters are not fully elucidated (Hutchison et al., 2016). This once again illustrates that the uptake systems of bacteria possess a broad spectrum and are not specific to one metabolite (Himmelreich et al., 1996). Several more complex organisms, including humans, have also lost the ability to synthesize the complete spectrum of proteinogenic amino acids by themselves and instead depend on nutritional uptake of the essential amino acids (Reeds, 2000). For human nutrition, nine amino acids have been labelled as essential amino acids, as they have to be taken up from food sources (Young, 1994), while the remaining amino acids can still be synthesized. Potential toxic effects of amino acids and their derivatives are also discussed in humans, as methionine is known to exacerbate psychopathological symptoms in schizophrenic patients, but no harmful long-term consequences could be observed in healthy probands (Garlick, 2006). Metabolic disorders in sulfur-containing amino acid metabolism can also lead to dangerous accumulation of methionine and cysteine (Kožich and Stabler, 2020). The tight interplay of amino acid processing enzymes therefore is necessary to control intracellular amino acid concentrations at all times. The total concentrations of free amino acids in the soil is estimated to be in the range of up to 0-100 µM (Jones, 2002), which is a much lower than the amino acid concentration used for the laboratory growth experiments. This implies that this unnaturally high concentration of amino acids is one of the reasons for the L-amino

acids to become toxic. Still, the question remains, why do numerous import mechanisms in *B. subtilis* occur without regulation, whereas the processes of export and synthesis are tightly regulated. One answer might be the fact that *B. subtilis* usually does not live solitarily but rather in a microbial community, where the favorable nutrients are embattled and scarce. Therefore, the quick uptake of amino acids is beneficial, to secure valuable nutrient sources. The major low affinity amino acid importer AimA is virtually not regulated and steadily expressed under most conditions. Expression analysis revealed no changes in expression even when supplementing the medium with serine or glutamate, or growing the cells in complex (LB) medium (Klewing, 2019). This indicates that the cells allow a constant flow-in of glutamate as well as other amino acids and then react to it, once it is within the cell. The steady uptake usually does not cause any problems, as glutamate and glutamine feed into ammonium production, which was shown to not be harmful for cells at even very high concentrations (Müller et al., 2006). Glutamate only poses a problem, when it cannot be broken down anymore (Commichau et al., 2008), similar to our findings with asparagine. Amino acids with a naturally low intracellular concentration could pose problems when they accumulate within the cell. One of the amino acids with a generally low intracellular concentration is histidine (Bennett et al., 2009). It is not completely clear, why histidine exerts the growth inhibiting effects on *B. subtilis*. Still, it is likely that histidine itself is causing this effect as the degradation products only feed into glutamate production (Chasin and Magasanik, 1968) and are not involved in any other intracellular reactions. Glutamate itself can also become toxic, when the glutamate dehydrogenases are absent. The deletion of RocG renders the strain sensitive to glutamate. This causes mutations, which decryptify GudB to still enable degradation of glutamate (Gunka et al., 2012; Belitsky and Sonenshein, 1998). The Δ*rocG* Δ*gudB* double deletion strain cannot utilize glutamate as single carbon source anymore and acquires mutations in the glutamate synthase gene *gltB*, to avoid the accumulation of glutamate (Commichau et al., 2008). Furthermore, there exists a link to asparagine/aspartate metabolism, as the cells also acquire mutations inactivating the *ansR* gene. This causes an overexpression of the aspartase pathway, which allows the efficient utilization of glutamate, even recovering the ability of the cells to utilize glutamate as sole C-source (Flórez et al., 2011). This again illustrates the link of glutamate to a multitude of other amino acid pathways. Glutamate is also known to indirectly increase the intracellular potassium concentrations by changing the affinity of the potassium importer KtrCD (Krüger et al., 2020). Our experiments with the Δ*dac* strain of *B. subtilis* raised the question whether amino acids, other than glutamate are able to increase the intracellular potassium concentration and thereby indirectly pose problems for the c-di-AMP free strain. Other amino acids could have a similar effect on potassium importers, or indirectly account for higher intracellular potassium concentrations, as they feed into glutamate production. The link between amino acid import and potassium import seems obvious as many amino acid transporters depend on potassium to even carry out their importing function

(Castagna et al., 1998). This also explains the lower rate of amino acid uptake in potassium depleted cells, as shown in multiple cases (Flynn and Syrett, 1986; Thompson and MacLeod, 1974). On the other hand, it was reported that the affinity of the glutamate and aspartate importer GltT was increased fivefold in high potassium concentrations (Krüger et al., 2021). Generally, this implies that more amino acids are taken up at high potassium concentrations. The higher intracellular concentrations then could pose a problem for the Δ*dac* strain, as it is not able to balance the osmotic equilibrium (Commichau et al., 2018; Bhowmick et al., 2023). All of this points towards a counterregulation of the two most abundant metabolites of the bacterial cell, which makes sense as they are able to level out the charge of the respective other (Gundlach et al., 2018). Finally, amino acids, especially D-amino acids may also be falsely integrated into the polypeptide chain during protein biosynthesis, which causes the formation of nonsense proteins and is lethal to the cell at higher concentrations. The reasons for amino acid toxicity are summarized in Fig. 17.

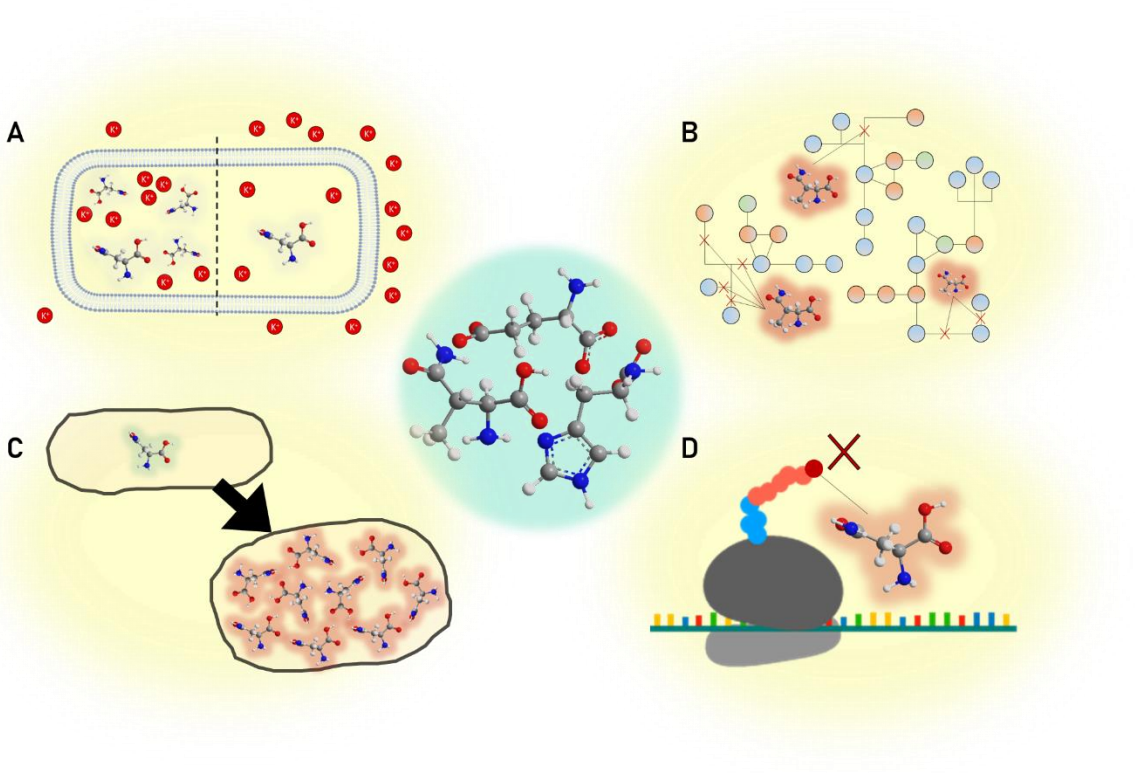

Figure 17 Reasons for amino acid toxicity. (A) Amino acids potentially increase intracellular potassium concentrations, which is especially harmful for the Δ*dac* strain of *B. subtilis*. **(B)** Amino acids interfere with other metabolic pathways due to their high reactivity. This leads to a potential shortage of metabolite endproducts. **(C)** Amino acid accumulation within the cell disrupts the osmotic balance within the cell. **(D)** Amino acids are falsely integrated into the polypeptide chain, which leads to formation of nonsense proteins.

5.2. AzlCD is a broad range amino acid exporter, AimA and BcaP are broad range importers

The results show that *B. subtilis* responds to amino acid pressure via enhanced export by the two component exporter AzlCD. While it was originally described as an exporter for the leucine analog, azaleucine (Belitsky et al., 1997), it now became evident, that AzlCD has to be considered as a general broad range exporter for amino acids. We could show that AzlCD also exports the two proteinogenic amino acids, histidine and asparagine. This occurs with enhanced efficiency by acquiring mutations in the transcriptional repressor AzlB. AzlB itself belongs to the Lrp protein family of transcriptional repressors. The name of this family derives back to *E. coli*, where Lrp was first described (Tuan et al., 1990). Here, the transcriptional repressor binds to L-leucine and acts as a major regulator protein, being involved in the regulation of about 75 genes (Lin et al., 1992). The protein forms octamers, with multiple DNA binding sites, as well as a ligand binding site in the center (los Rios and Perona, 2007). This structure is conserved amongst bacteria, and AzlB of *B. subtilis* shares very high structural similarity with Lrp of *E. coli*. Therefore, it is to be assumed, that *azlB* also forms octamers, as shown in Fig. 18.

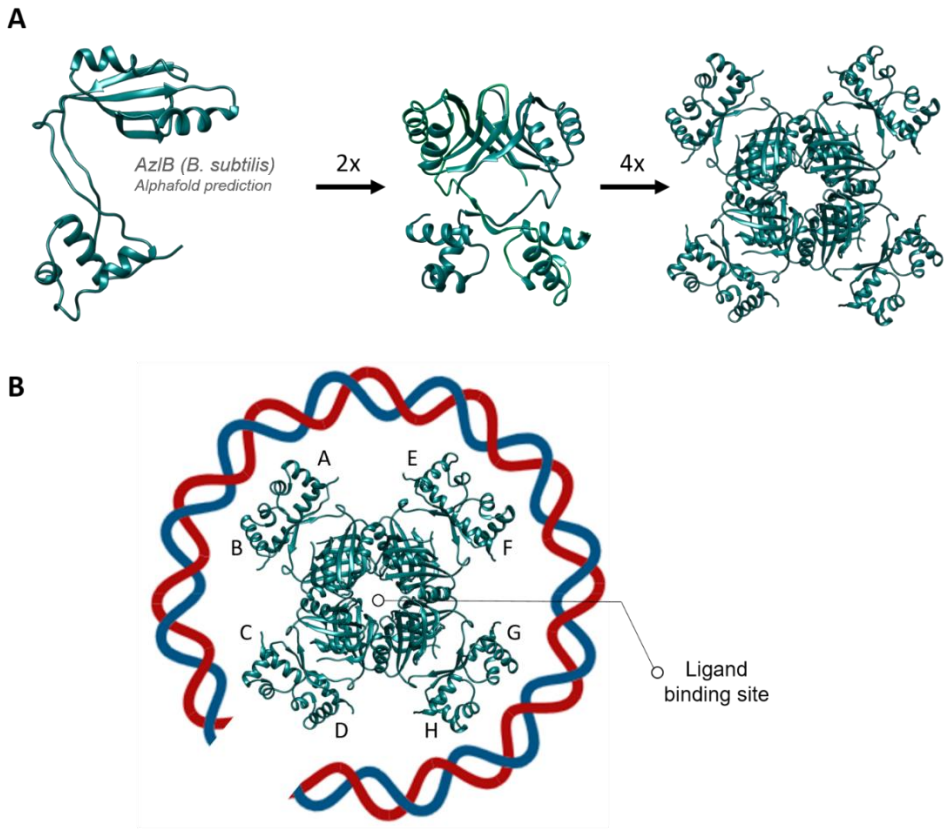

Figure 18 Multimerization of AzlB. A Visualization of octamer formation of AzlB. **B** The AzlB octamer has 8 DNA binding sites, as well as a ligand binding side in the middle (Figure adapted from (los Rios and Perona, 2007)).

The multimerization of AzlB is likely hindered in the suppressor mutants, in a way that it does not bind to the DNA anymore as usual, which causes a decrease in the repressive effect. It is questionable, whether efficient multimerization of AzlB is still possible, although the mutated variants of *azlB* still do not fully restore growth under histidine pressure. This indicates a weakened, but not missing activity of AzlB in these suppressor mutants. The complete absence of AzlB in the deletion mutants then causes steady overexpression of the downstream genes in the operon, which fully rescues the growth. BrnQ, a transporter for valine and isoleucine is also part of this operon. Although its role in acquiring resistance to both histidine and asparagine could be excluded by the Δ*dac* Δ*azlBCD* and Δ*ansAB* Δ*ansZ* Δ*azlBCD* mutants, it is still notable that the operon carries both, an importer and an exporter, simultaneously. This also makes it likely that AzlCD, while being a broad range amino acid exporter is not exporting branched chain amino acids, as this would counteract the activity of BrnQ. AzlB itself is the first gene in the operon and unlike other regulators part of the of the operon it regulates (Belitsky et al., 1997). This is particularly interesting, as the normal functionality of the operon therefore is to be understood as a short-lived event. When the operon is induced, the broad range export of amino acids is occurring with high efficiency via AzlCD. Simultaneously, the expression of *azlB* also causes the operon to be repressed again, causing the system to come to a halt again. This prevents the unwanted loss of amino acids. Still, it is puzzling that none of the exported metabolites or other amino acids induce the operon. While it is likely that the inducer of the operon just was not found yet, this also raises the hypothesis that *B. subtilis* deliberately acquires mutations in *azlB*, but only at lethal stressor conditions. Such a strategy would very rarely occur in a natural setting as the export of L-amino acids usually is very unfavorable. This might be the reason, why it is regulated in this way and *B. subtilis* relies on mutations to activate the broad range export of amino acids. As all of our suppressors carried mutations in *azlB*, while either histidine or asparagine was applied, this has to be considered as one of the first options of *B. subtilis* to deal with amino acid stress. This also raises the hypothesis, that import of these two amino acids is split between multiple uptake transporters, with no major transporter, as the importers would otherwise have been affected during the suppressor screening process. Previous studies of amino acid transporters have revealed that even when multiple importers of an amino acid were knocked out, it still is able to enter into the cell (Klewing et al., 2020). The toxic amino acid serine was still able to enter the cell, when three importers (AimA, YbxG, BcaP) were deleted and thus still exerted its toxic/growth inhibiting effects. This uptake works by transporters with a low affinity for serine, which only take it up under this specific condition and at very high concentrations. This suggests that there is no real end point for uptake research, as there is no strain in *B. subtilis*, which is not able anymore to take up a specific amino acid, even if the main importers are deleted. Thus, finding out the main importers is of most physiological relevance. This widespread ability of amino acid uptake is shared between bacteria. For *E. coli*, four known glutamate uptake transporters are described

(Schellenberg and Furlong, 1977). Still, the presence of multiple transporters is no guarantee for their uptake efficiency, as wild type cells of *E. coli* are not able to utilize glutamate as their sole source of carbon (Halpern and Umbarger, 1961). In this case, mutations that increase the glutamate uptake activity are needed in order for the cells to grow with glutamate as sole carbon source (Halpern and Lupo, 1965). The uptake systems of *B. subtilis* are sufficient to allow it to grow on multiple individual amino acids as sole carbon source. This also includes histidine as well as asparagine, as they both are degraded to glutamate, which itself can be used as sole carbon source (Stannek et al., 2015). Just recently, another link between aspartate and glutamate was discovered. It was shown that the glutamate auxotrophic Δ*gltAB* mutant could overcome this deficiency by acquiring mutations affecting *ansR*, coding for the transcriptional repressor of the *ansAB-mleNA* operon, as well as *citG*, coding for fumarase, respectively (Mardoukhi et al., 2023). This allows enhanced glutamate production from aspartate, which has to be present in much lower concentrations within the cell than glutamate. Asparagine, which is directly degraded to aspartate via AnsA, also helps in this matter. Our experiments uncovered the mechanisms of L-asparagine import into the cells, as AimA and BcaP are both contributing to the uptake of L-asparagine. This is illustrated by the fact, that the Δ*aimA* deletion in the Δ*dac* background provides the cells with L-asparagine resistance. In the other L-asparagine sensitive genotype the broad range importer BcaP was mutated, specifically in the Δ*ansAB* Δ*ansZ* Δ*aimA* Δ*azlBCD* deletion strain, where the possibility of adaptation via enhanced export was excluded. The resulting suppressors can deal with any concentration of L-asparagine that was added to the medium, as also shown by the Δ*ansAB* Δ*ansZ* Δ*aimA* Δ*azlBCD* Δ*bcaP* deletion strain. It is likely that no further suppressor mutants can develop from this strain, as the asparagine tolerance is already so high. The histidine uptake in *B. subtilis* still remains unsolved. Specifically, only HutM was suggested to be involved in histidine uptake, as it is part of the *hut* operon in *B. subtilis* (Wray and Fisher, 1994). However, no studies were performed with it, and it was never found during any of our suppressor screens. The carried out experiments exclude AimA to be involved in histidine uptake, as experiments with the Δ*dac* Δ*aimA* strain showed no resistance to histidine. This suggests the existence of other histidine uptake transporters, which are yet to be characterized. One of our suppressors (GP3588) carried a mutation in the glutamate and aspartate transporter GltT. More conclusive studies are needed to elaborate its potential role in histidine uptake, although a few hints already point towards GltT being a broad range amino acid transporter as well. It is for example involved in the uptake of the total-herbicide glyphosate, as well as glufosinate (Wicke et al., 2019), which both are toxic analogs to glutamate. Our results also point towards an influence of *ydeC* in asparagine and serine resistance. YdeC is a transcription factor and the acquired mutations most likely change the regulation of its corresponding gene *ydeD*, which codes for an exporter and is therefore causing enhanced export of

amino acids like L-Asn and L-Ser. Again, the export is coupled to mutations waiting to happen, which is a similar concept to what we encountered with AzlB.

5.3. D-amino acids

Our experimental setup with D-asparagine led to suppressor mutations in MleN, which block the import of the toxic D-asparagine. As MleN is expressed in an operon together with the asparaginase and aspartase genes *ansA* and *ansB*, it was tempting to speculate that it plays a role in asparagine uptake. However, our experiments could show that MleN presumably only takes up D-asparagine into the cell, while not being involved in L-asparagine import, as the deletion mutant in the Δ*dac* background does not provide the cells with L-asparagine resistance. Although the role of MleN in L-asparagine uptake cannot be fully excluded, it was never found in any of the suppressor screens and therefore most likely does not function as main uptake transporter for L-asparagine, as this role is attributed to AimA and BcaP. MleN itself was firstly described as a malate/lactate antiporter, which makes physiological sense, as the whole operon feeds into the TCA. The malate that is taken up by MleN can be utilized by MleA, which leads to pyruvate production. However, the uptake of asparagine also would be reasonable, as the asparaginase and aspartase are also expressed in the same operon. A D-asparaginase function of AnsA cannot be excluded and there are reports of D-asparaginases, which task is most like to fuel the TCA, by producing fumarate (Guy and Daniel, 1982). Therefore, the uptake of D-Asn by MleN might not be fully accidental and deserves further investigation. MleN itself is also not well conserved amongst bacteria, as there is no significant homolog in *E. coli*. However, the other malate importer of *B. subtilis*, YflS is conserved and has homologs in *S. aureus*, *C. glutamicum* and *E. coli* as well. YflS is induced by malate and is likely to be more specific than MleN, as no suppressor mutations were found, when applying D-Asn stress. Consequently, one cannot assume malate importers to be importers for D-Asn as well in every case, although the two metabolites are structurally very similar (Fig 19), although the possibility of D-Asn import via YflS cannot be fully excluded.

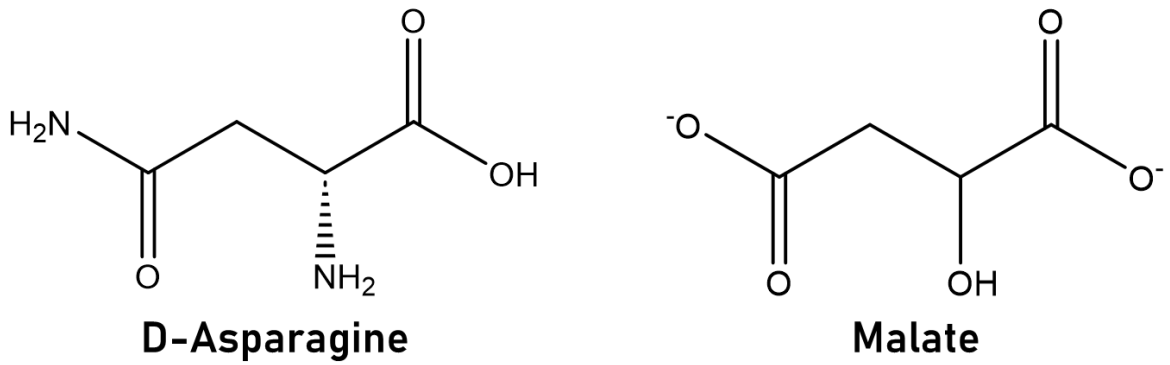

Figure 19 Structural similarity between D-asparagine and malate. Both are taken up by the malate/lactate antiporter MleN.

Still, it would be interesting to see, if the malate transporters of other bacteria are also responding to D-Asn. Another question is whether more substrates than malate and D-Asn are imported by MleN, as antiporters in *B. subtilis* possess a flexible structure, allowing them to take up multiple substrates (Krulwich et al., 1994). Generally, also other types of transporters are not limited to only one substrate. The gamma-amino butyric acid permease GabP, for instance, also has a slight affinity for L-proline (Zaprasis et al., 2014). One example, where the uptake of D-amino acids happens via the same transporter as the L-counterpart was described in *B. subtilis* for alanine, where the permease AlaP takes up both: L- and D-alanine, with a higher specificity towards D-alanine (Sidiq et al., 2021). This is not surprising, as D-alanine is an integral component of the cell wall and transport of it was also described earlier in *B. subtilis* (Clark and Young, 1974), as well as other bacteria (Lombardi and Kaback, 1972; Short et al., 1972). *B. subtilis* even possesses the essential Alanine racemase Alr, which is constantly converting L-alanine to D-alanine. Without it, the cells lyse quickly in complex medium (Heaton et al., 1988). A deletion of the *alr* gene is only possible if the alanine transporter AlaP is still present and the medium is supplemented with excess D-alanine (Sidiq et al., 2021). For production of the other cell wall component, D-glutamate, *B. subtilis* relies on two glutamate racemases, YrpC and RacE, with the latter being essential. This marks a difference to the closely related *B. anthracis*, which also possesses two glutamate racemases but none of them are individually essential, only the double mutant is not viable anymore (Oh et al., 2015). The essentiality of the D-amino acid acquiring genes is intriguing and potentially the result of a generally lower concentration of D-amino acids in the environment or transporters with too little affinity and therefore inefficient uptake of D-amino acids. While there exist bacteria, which efficiently take up D-glutamate from the environment, and even using it as their sole C source (Naganuma et al., 2018), this is not possible for *B. subtilis*. This is particularly interesting, since the transporters for glutamate in *B. subtilis*, GltT and AimA, both are broad range importers, which are not restricted to only glutamate. Further studies with those two and other candidates could reveal the identity of a D-glutamate transporter, but for now, simultaneous uptake of L- and D-amino acids was not reported for any transporter other than AlaP in *B. subtilis*. In *E. coli* however, the general amino acid permease for aromatic amino acids AroP is capable of taking up both D- and L- variants of tyrosine, tryptophan and phenylalanine (Kuhn and Somerville, 1974). This is striking, as the best homolog for the *E. coli* AroP is AlaP in *B. subtilis*, with an identity score of 42%. Structural experiments with AroP and AlaP therefore could uncover the features that are needed for a transporter to be able to transport L- and D-amino acids simultaneously. The affinity for D-amino acids might be much lower than the affinity for L-amino acids, which would mean that they compete for the import- a common event in transporter physiology. Competitive uptake assays carried out by (Manuel Königshof, 2022) with high amounts of malate under D-asparagine pressure showed, that D-asparagine still entered into the cell and exerted its toxic effect. The naturally occuring concentrations of D-

asparagine are much lower than the concentrations we used to isolate suppressor mutants. Still, it is notable, that *B. subtilis* tolerates D-asparagine concentrations up to 5 mM until it becomes toxic and the suppressor formation starts. The *ansAB-mleNA* operon is also slightly induced by not only L-asparagine, but D-asparagine as well. This could imply a D-asparaginase activity to be present in *B. subtilis*, potentially also carried out by AnsA. Still, the preferred adaptation route of *B. subtilis* is the disruption of D-asparagine import and no hyperactive AnsA mutants were obtained during the suppressor screening process. Dismantling of intracellular D-asparagine could also be possible by racemization via RacX. Therefore, the normally carried out reaction from L-asparagine to D-asparagine would have to work bidirectionally, so that the exuberance of D-asparagine could be disarmed by the racemase. However, experiments with an overexpressed RacX (data not shown) did not yield enhanced resistance to D-asparagine pressure. Gradual secretion of D-Asn into the environment could also be a coping mechanism. The export mechanisms might be more active during stationary phase, which was reported for *B. subtilis* (Lam et al., 2009). Lastly, reactivation of the *dtd* gene, coding for D-aminoacyl-tRNA deacylase in the wildtype could foster *B. subtilis* handling the D-amino acid stress. This gene is responsible for the prevention of misincorporation of D-amino acids, but is not expressed in the laboratory wild type strain 168 (Leiman et al., 2013). All in all, this work can be seen as a starting point regarding D-amino acid research, with more to come in the future.

5.4. Advances in amino acid metabolism

B. subtilis is one of the most extensively studied model organisms. One of the main goals of our research was to uncover mechanisms and principles that could be transferred to other organisms as well. The field of amino acid metabolism is particularly interesting in this regard, as individual amino acids are a popular product in biotechnology and the understanding of basic essential metabolic principles also drives the understanding about minimal life requirements further. The Mini*Bacillus* project aimed to create the smallest possible genome of a functional *B. subtilis* cell (Reuß et al., 2016). For such a task to be realized, it is helpful to uncover the exact functions of uncharacterized proteins. The majority of essential genes are involved in protein synthesis and general metabolism (Kobayashi et al., 2003; Commichau et al., 2013), while genes involved in amino acid metabolism can be deleted and therefore are described as non-essential. In fact, none of the amino acid transporters are essential in *B. subtilis*, and the synthesis genes are also dispensable, with a few exceptions. AroE, AsnB and DapG (Koo et al., 2017) are all essential due to their secondary functions and not because of their ability to synthesize amino acids. A few amino acid synthesizing genes are also described to be “co-lethal”, as their knockout in combination with another deletion would be lethal to the cells. This is the case for the *glnR-glnA* pair, as well as the *tyrA-hisC* pair (Tanaka et al., 2013). This illustrates that a lot of genes involved in amino acid metabolism might have secondary functions that are not well understood yet.

One also has to keep in mind that the knockout of the synthesis genes is only possible on complex medium, as all of the synthesis genes have to be present in a minimal medium setting. A big area of research was designated to characterize the highly expressed genes in *B. subtilis*, as they are carrying out the most important tasks in the cell. A majority of the highly expressed genes are essential genes, which make up the largest fraction of the proteome, as they account for 57% of translation resources (Reuß et al., 2017). Still, there are over 40 highly expressed proteins in *B. subtilis* with unknown function. While following this approach, proteins like AzlB often go under the radar, as they require specific conditions to even be moderately expressed. Furthermore, it is important to keep in mind that the overall expression profile of *B. subtilis* drastically changes upon sporulation (Phillips and Strauch, 2002). The role of amino acids in sporulation are diverse, as omission of leucine, tryptophan or threonine in the auxotrophic mutants leads to direct inhibition of sporulation (Doering and Bott, 1972). However, threonine was also reported to inhibit sporulation, due to its detrimental effects on valine synthesis (Lamb and Bott, 1979a), indicating that the balance of amino acid concentrations is essential for sporulation as well. Generally, the hierarchy of the mutations that occur, when *B. subtilis* is subjected to amino acid stress is to be pointed out. The COG database (Galperin et al., 2021) is another approach to analyze reoccurring systems, as it lists conserved genes between bacteria and thereby illustrates the importance of also lesser expressed genes. Of 1309 organisms in the library, *azlB* has homologs in 1015 of them, which proves that the inisghts gained from our experiments also has significance in a bigger picture. In fact, export of branched chain amino acids is also regulated via Lrp, a protein of the same family in *C. glutamicum* (Lange et al., 2012). In this case, Lrp is an activator of the exporter complex, and requires the presence of amino acids to become active. This shows that the principle of Lrp-family-protein mediated export is well conserved amongst bacteria. In *B. subtilis*, also other variants of Lrp proteins exist. Two transcriptional regulators, LrpA and LrpB are likely involved in the repression of *glyA* (Dartois et al., 1997), thus controlling glycine biosynthesis. LrpC of *B. subtilis* is involved in DNA repair mechanisms (Tapias et al., 2000), which illustrates the multitude of functions carried out by Lrp-proteins. This also could mean that more secondary functions of AzlB could be uncovered in the future. Adding to this, there are also other examples for important systems that are normally not expressed and only act when the specific condition is met. A good example for this are the amino acid utilization enzymes. Usually, *B. subtilis* is able to manage the degradation of amino acids via an array of transcription factors, which are often amino acid specific. In case of histidine, the utilization is controlled by the transcriptional antiterminator HutP (Dhakshnamoorthy et al., 2013), which directly responds to histidine and allows its degradation by the proteins encoded in the *hut* operon. The degradation of asparagine is also controlled by the asparagine specific repressor *ansR* (Sun and Setlow, 1993), which controls the *ansAB-mleNA* operon. Mutations in *ansR* would result in an overexpression of the operon, causing efficient asparagine degradation. While it is logical, that such

mutations were not found in the background of the Δ*ansAB* Δ*ansZ* double deletion mutant, it also was unaffected in the Δ*dac* background with L-Asn and wild type, when D-Asn was supplemented. This could be due to the fact that the reaction products downstream of the asparagine degradation- especially glutamate (Krüger et al., 2021) are also harmful to the Δ*dac* strain of *B. subtilis*, and therefore mutation of the importers is the easiest option. The mutations in the Δ*ansAB* deletion strain differ between the wild types 168 and SP1, as either *azlB* or *aimA* were target of the adaptative mutations. The hierarchy here is not fully clear, although the main goal of the cells is avoidance of the stressor via disrupting import or upregulating export. The differences in the genomes (Richts et al., 2020) could cause *aimA* to be more easily mutated in the SP1 strain. Additionally, the suppressors originate from different media, with LB being also rich in glutamate. The decreased import of glutamate therefore could benefit the Δ*ansAB* deletion strain. A higher quantity of analyzed suppressors could also reveal more uniform results and could be investigated. Still, both of our main experimental endeavors yielded increased export as the first hierarchical option to cope with amino acid stress. The field of amino acid exporters is a novel field of research for *B. subtilis*. While it has been shown that the export of L-amino acids like L-glutamate, L-lysine, L-isoleucine and L-threonine is actively carried out by *C. glutamicum* as well as *E. coli* (Krämer, 1994; Eggeling and Sahm, 2003), *B. subtilis* was not considered as amino acid exporter organism. Still it finds many uses as industrial workhorse. One example for this is the production of its asparaginase, which is isolated to catalyze the reaction of L-asparagine to L-aspartate. (Pourhossein and Korbekandi, 2014). This is of great commercial interest, as it is clinically used, specifically in treatment of lymphoblastic leukemia (Zuo et al., 2015). The asparaginase also finds use in the food industry, where it prevents the formation of acrylamide, as asparagine is eliminated as the main precursor here (Hendriksen et al., 2009). This illustrates the desirable optimization of enzyme production, which is only possible by increasing knowledge (Feng et al., 2017). Just recently, a strain of *C. glutamicum* was engineered to increase the production of β-alanine (Ghiffary et al., 2022). The *B. subtilis* L-aspartate-α-decarboxylase *panD* gene was used for this purpose, as it showed the most efficient synthesis. The possibility of genome editing via CRISPR-Cas9 further enhanced the utility of *B. subtilis* in a biotechnological aspect (Altenbuchner, 2016). Gathering specific knowledge on amino acid importers as well as exporters, like AimA, BcaP and AzlCD could provide diverse fields of action in biotechnology, as these transporters could also be expressed in another organism to harvest amino acids. A lot of metabolic processes, especially amino acid synthesis, function via negative feedback loops, which have to be identified by research and then removed to optimize the production (Lee and Kim, 2015). This work as well as many other studies will enhance the understanding of amino acid metabolism and how we can use it further.

5.5. Outlook and open questions

Our work provided some insights into the field of amino acid research. Still, a lot is to be uncovered in the future. To uncover further insights of histidine uptake, a Δ*hutH* Δ*azlCD* mutant could be created. This would prevent histidine degradation, as well as the adaptation possibility via the exporter. The accumulation of histidine is likely to be toxic for the cells, so that suppressor mutants could be isolated. Furthermore, AimA is likely to not be involved in histidine uptake, so potentially other broad range importers like BcaP or GltT are involved in its uptake, as already hinted by one of our suppressor mutants. The role of HutM in histidine uptake could also be verified, if its deletion provides enhanced histidine resistance. Other amino acids with no known transporter are phenylalanine and tyrosine. However, it was suggested before that both enter the cell via the same uptake transport system (D'Ambrosio et al., 1973). Both amino acids are only present in very low concentrations within the cell (Bennett et al., 2009). Therefore, an accumulation of them could quickly disturb the standard cellular flow. Furthermore, no pathway is known for the designated degradation of either of these two amino acids, and they also do not take part in other intracellular reactions. This implies that *B. subtilis* likely only possesses low-affinity transporters for the two of them, to prevent uncontrolled accumulation. This also could mean, that tyrosine and phenylalanine do not have a main importer that is to be identified, but rather are a “by-catch” of broad range amino acid importers. Glycine is the simplest amino acid, talking from a structural perspective. It is also the only amino acid without a stereoisomeric center, which consequently means no D-isomeric form for the potential isolation of suppressor mutants. Still, high amounts of glycine could be toxic for the cell, especially if the degradation pathways are deleted. Glycine feeds into glyoxylate production, which was shown to play a role in sporulation onset of *Bacillus species* (Megraw and Beers, 1964). Given its small size, it could also be a secondary substrate or even be taken up by non-designated amino acid transporters. Further studies also have to take into account that the general broad range amino acid transporters are already identified. AimA, BcaP and GltT should be checked primarily for the import of amino acids or other metabolites, before diving deeper into transport mechanisms, as these three showed up in numerous suppressor screens in the past. Experiments with radiolabelled metabolites or directed structural analysis via Cryo EM or similar processes could uncover the full spectrum of importable metabolites for these three importers. Since amino acid transporters are so ambiguous, one could also think of a rescue experiment, where the competition between the two imported substrates could provide beneficial effects for the growth, when less of the toxic substrate is taken up. In our case, we performed an experiment with simultaneous supplementation of L-Asn, as well as other amino acids in the Δ*ansAB* Δ*ansZ* double deletion strain. Here, facilitated growth was observed, when certain amino acids were added (data not shown). The next step would be the deletion of importers, which import L-Asn, to see, if the rescue still takes place, or if it is dependent on the uptake via the shared importer. The approach with rescuing

metabolites therefore deserves a deeper look and experimental setup. Our experiments with the Δ*dac* strain often revealed a potential link between amino acids and the intracellular potassium concentration. While it was already published for glutamate (Krüger et al., 2020) to play a role in this matter, the other amino acids also deserve to be analyzed. They might not only replace potassium in its role of buffering the negative charge of the DNA (Gundlach et al., 2017a), but also increase intracellular potassium concentrations in other ways. Metabolomic studies with measurements of potassium concentration could uncover this role further. It is also likely that *B. subtilis* possesses not only a multitude of amino acid importers, but exporters as well. The regulation of these exporters could work in a similar manner as AzlCD. So far, the exporters might have existed "under the radar", as their general expression is very low. However, mutations in regulator genes could elevate the expression of the exporters and allow efficient export. The proteins of the EamA-transporter family therefore need to be analyzed further. It could also be the case that some exporters are induced by the substrates that they are exporting, without a mutation to be necessary to become active. Expression analysis of the remaining importer and exporters therefore could yield valuable insights over the substrates they are transporting. Finally, the field of D-amino acid im- and export deserves a deeper look. A complete study over which D-amino acids are harmful to *B. subtilis* could uncover novel transporters, as well as metabolic pathways to cope with D-amino acid stress. D-glutamate in particular is likely to be taken up by *B. subtilis*, due to its role in cell wall composition. To uncover the uptake, mutant strains of RacE and YrpC could be created, while supplementing D-glutamate to the medium. If the creation of the double mutant is possible, then this would imply the efficient uptake of D-glutamate via unknown transporters. Overexpression plasmids carrying characterized and uncharacterized transporters could show, whether the uptake is facilitated in some cases and thus uncover the uptake transporter for D-glutamate in *B. subtilis*. The export of D-amino acids seems reasonable, although AzlCD as broad range exporter seemingly is not involved in the export process. Still there are numerous uncharacterized exporters in *B. subtilis*, which should be further investigated. Expression analysis as well as growth experiments with overexpressed transporter variants could shed a light on their functionality within the cell.

Chapter 6 – References

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Chapter 7 – Appendix

7.1. Materials

7.1.1. Chemicals

Agar	Roth, Karlsruhe
Agarose	Biozym Scientific GmbH, Hessisch Oldendorf
Antibiotics	Merck, Darmstadt
Amino acids	Merck, Darmstadt
β-Mercaptopethanol	Merck, Darmstadt
Bacto agar	Becton, Dickinson and Company, Heidelberg
DMSO	Roth, Karlsruhe
dNTPs	Roche Diagnostics, Mannheim
HDGreen™	Intas, Göttingen
Nutrient broth	Merck, Darmstadt
ONPG	AppliChem, Darmstadt
Yeast extract	Oxoid, Heidelberg

Further chemicals were purchased from Carl Roth, Merck, Peqlab or Sigma-Aldrich.

7.1.2. Enzymes

DNase I	Roche Diagnostics, Mannheim
DreamTaq DNA Polymerase	ThermoFisher, Waltham, USA
FastAP	ThermoFisher, Waltham, USA

7.1.3. Commercial systems

NucleoSpin Plasmid-Kit	Macherey-Nagel, Düren
peqGOLD Bacterial DNA Kit	PeqLab, Erlangen
QIAquick PCR purification Kit	Qiagen, Düsseldorf
Rneasy Plus Mini Kit	Qiagen, Düsseldorf

7.1.4. Instruments/ Equipment

Autoclave	Zirbus technology, Bad Grund
Biofuge fresco	Amersham, Freiburg
ChemoCam imager	Intas, Göttingen

Electronic scale	Sartorius, Göttingen
GelDoc™ XR+	Bio-Rad, München
Gel electrophoresis device	Waasetec, Göttingen
Incubator Innova R44	New Brunswick, Neu-Isenburg
Incubator shaker Innova 2300	New Brunswick, Neu-Isenburg
LabCycler	LabCycler SensorQuest, Göttingen
Magnetic stirrer	JAK Werk, Staufen
Microplate reader SynergyMx Mini-Protean	BioTek, Bad Friedrichshall
Mikroprozessor pH-Meter 766 Calimatic	Knick, Berlin
Nanodrop ND-1000	ThermoFisher, Waltham, USA
Open air shaker Innova 2300	New Brunswick, Neu-Isenburg
Refrigerated centrifuge PrimoR	Heraeus Chris, Osterode
Scale Sartorius universal	Sartorius, Göttingen
Special accuracy weighing machine	Sartorius, Göttingen
Spectral photometer Ultraspec 2000	Amersham, Freiburg
Stereo Lumar V12 stereo microscope	Carl Zeiss, Göttingen
Sterile bench Hera Safe	ThermoFisher, Waltham, USA
Thermocycler	Biometra, Göttingen
UV Transilluminator 2000	Bio-Rad, Munich
Vortex	Bender and Hobein, Buchsal
Water desalination plant	Millipore, Schwalbach

7.1.5. Materials

96-well plates	Sarstedt, Nümbrecht
Cuvettes (microliter, plastic	Sarstedt, Nümbrecht
Falcon tubes (15 ml, 50 ml)	Sarstedt, Nümbrecht
Gene Amp Reaction tube (PCR)	Perkin Elmer, Weiterstadt
Glass pipettes	Brandt, Wertheim
Microlitre pipettes (2 µl, 20 µl, 200 µl, 1 ml)	Gilson International, Berlin
5 ml pipette	Eppendorf, Hamburg
Petri dishes	Greiner, Nürtingen
Pipette tips	Sarstedt, Nümbrecht
Reaction tubes	Greiner, Nürtingen
Single-use syringes	Becton Dickson, Drogheda, Ireland

7.1.6. Software and webpages

Program	Provider	Application
AlphaFold	(Jumper et al., 2021)	Prediction of protein structures
AlphaFold Multimer	(Evans et al., 2021)	Structural prediction of protein complexes
BLAST NCBI	National institute of Health, USA	BLAST searches
Citavi	Swiss Academic Software GmbH	Reference manager
***Core*Wiki**	Department for general microbiology University of Göttingen	Metabolite reaction pathways in *B. subtilis*
Foxit Reader	Foxit Software	Literature viewer
Gen5™ Data Analysis Software	BioTek®	Plate reader analysis
Geneious Prime 2021.0.3	Biomatters	DNA analysis
ImageLab™ Software	BioRad	Geldoc imaging
Microsoft Office 365	Microsoft Inc.	Data processing and writing
Pymol 2.0	Schrödinger LLC	Protein structure visualization
***Subti*Wiki 4.0**	(Pedreira et al., 2022)	*B. subtilis* database

7.2. Bacterial strains

7.2.1. *E. coli* strains

Name	Genotype	Reference/ Construction
DH5α	*recA1 endA1 gyrA96 thi hsdR17rκ- mκ⁺relA1 supE44 Φ80ΔlacZΔM15 Δ(lacZYA-argF)U169*	(Sambrook et al., 1989)

7.2.2. *B. subtilis* strains

Name	Genotype	Reference/ Construction
168	*trpC2*	Laboratory collection
GP314	*trpC2 amyE*::(P_{pgi}-*lacZ cat*)	(Ludwig et al., 2001)
GP829	*trpC2 amyE::(ansA-lacZ aphA3)*	pGP388 → 168
GP983	*trpC2* Δ*cdaS*::ermC	(Mehne et al., 2013)

GP997	*trpC2* Δ*cdaA*::cat	(Mehne et al., 2013)
GP1155	*trpC2 amyE::(ansA-lacZ cat)*	pGP872 → 168
GP1460	*trpC2* Δ*mleN*::*spc*	VK3 → 168
GP2032	*trpC2* Δ*cdaS::ermC ΔcdaA::cat*	(Gundlach et al., 2015)
GP2222	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*disA*::tet	(Gundlach et al., 2017b)
GP3054	*trpC2* Δ*cdaS::ermC* Δ*disA::tet* Δ*cdaA::cat* Δ*aimA::phleo*	(Krüger et al., 2021)
SP1	wild type, *trpC*$^{+}$	(Richts et al., 2020)
VK3	BD99 Δ*mleN*::spc	(Wei et al., 2000)

7.2.3. Strains constructed in this work

Name	**Genotype**	**Construction**
GP1153	*trpC2* Δ*ansAB::ermC*	LFH → 168
GP2142	*trpC2* Δ*disA*::tet	LFH → 168
GP2715	*trpC2* Δ*disA*::spc	LFH → 168
GP2782	*trpC2* Δ*disA*::kan	LFH → 168
GP3588	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*disA*::tet *azlB* $_{\text{fs(pos 126 +T)}}$ *ktrD* $_{\text{fs(pos 72 –T)}}$ *kimA* $_{\text{fs(pos 128 +GTCGCAT)}}$ *gltT* (Thr342 Trp)	Suppressor of GP2222 (30mM His)
GP3590	*trpC2* Δ*aimA::phleo*	LFH → 168
GP3600	*trpC2* Δ*azlB*::spec	LFH → 168
GP3601	*trpC2* Δ*azlBCD*::kan	LFH → 168
GP3604	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*azlBCD*::kan	GP3601 → GP2032
GP3605	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*azlB*::spec	GP3600 → GP2032
GP3606	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*disA*::spec Δ*azlBCD*::kan	GP2715 → GP3604
GP3607	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*disA*::kan Δ*azlB*::spec	GP2782 → GP3605

GP3611	*trpC2 amyE*::(P_{yrdF}-*lacZ aphA3*)	pGP3807 → 168
GP3612	*trpC2 amyE*::(P_{azlB}-*lacZ aphA3*)	pGP3808 → 168
GP3613	*trpC2* Δ*azlB*::spec *amyE*::(P_{yrdF}-*lacZ aphA3*)	pGP3807 → GP3600
GP3614	*trpC2* Δ*azlB*::spec *amyE*::(P_{azlB}-*lacZ aphA3*)	pGP3808 → GP3600
GP3615	*trpC2* Δ*gltR*::spec	LFH → 168
GP3617	*trpC2* Δ*gltR amyE*::(p_{azlB}-*lacZ aphA3*)	pGP3808 → GP3615
GP3622	*trpC2* Δ*azlCD*::kan	LFH → 168
GP3623	*trpC2* Δ*azlBCD*::spec	LFH → 168
GP3625	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*azlBCD*::spec	GP3623 → GP2032
GP3626	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC *Δazl*Δ*BCD*::spec *ganA*::(p_{degQ36}-*azlC* aphA3)	pGP3811 → GP3625
GP3627	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*azlBCD*::spec *ganA*::(p_{degQ36}-*azlCD* aphA3)	pGP3812 → GP3625
GP3638	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*disA*::tet *azlB* (Asn24 Ser)	Suppressor of GP2222 (15mM His)
GP3639	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*disA*::tet *azlB* $_{fs}$ (pos 37 +CATTAATG)	Suppressor of GP2222 (15mM His)
GP3642	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*disA*::tet Δ*azlBCD*::spec *ganA*::(p_{degQ36}-*azlCD* aphA3)	GP2142 → GP3626
GP3643	*trpC2* Δ*cdaA*::cat Δ*cdaS*::ermC Δ*disA*::tet Δ*azlBCD*::spec *ganA*::(p_{degQ36}-*azlC* aphA3)	GP2142 --> GP3627
GP4158	*trpC2 mleN* $_{fs}$ (pos 517 + GC)	Suppressor of 168 (10 mM D-Asn)
GP4159	*trpC2 mleN* $_{fs}$ (pos 517 + GC)	Suppressor of 168 (10 mM D-Asn)
GP4173	*trpC2* Δ*cdaS::ermC* Δ*cdaA::cat* Δ*mleN::spec*	GP1460 --> GP2032

GP4177	*trpC2* Δ*cdaS::ermC* Δ*cdaA::cat* Δ*disA::kan* Δ*mleN::spec*	GP2782 --> GP4173
GP4191	*trpC2* Δ*mleN::spec amyE::(ansA-lacZ aphA3)*	pGP872 --> GP1460
GP4197	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan*	GP4229 --> GP1153
GP4202	*trpC2* Δ*hutH*::spec	LFH → 168
GP4205	*trpC2* Δ*hutH*::spec Δ*cdaA*::cat Δ*cdaS*::ermC	GP4202 → GP2032
GP4206	*trpC2* Δ*hutH*::spec Δ*cdaA*::cat Δ*cdaS*::ermC Δ*disA*::kan	GP2782 → GP4205
GP4229	*trpC2* Δ*ansAB:cat*	LFH → 168
GP4230	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan azlB* $_{N24S}$	Suppressor of GP4197 (5 mM L-Asn)
GP4231	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan azlB* $_{fs\ (pos\ 37\ +CATTAATG)}$	Suppressor of GP4197 (10 mM L-Asn)
GP4232	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan azlB* $_{Q129 \rightarrow stop}$	Suppressor of GP4197 (15 mM L-Asn)
GP4233	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan azlB* $_{D19G}$	Suppressor of GP4197 (30 mM L-Asn)
GP4236	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan* Δ*azlB::spec*	GP3600 --> GP4197
GP4237	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan* Δ*azlBCD::spec*	GP3623 --> GP4197
GP4239	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan* Δ*aimA::phleo*	GP3590 --> GP4197
GP4245	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan* Δ*azlBCD::spec* Δ*aimA::phleo*	GP3590 --> GP4237
GP4251	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan* Δ*azlBCD::spec* Δ*aimA::phleo ydeC*$_{H43Q}$ *bcaP*$_{A68E}$	Suppressor of GP425 (L-Asn gradient)
GP4252	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan* Δ*azlBCD::spec* Δ*aimA::phleo bcaP**	Suppressor of GP425 (L-Asn gradient)
GP4266	*trpC2* Δ*bcaP::cat*	LFH → 168

GP4267	*trpC2* Δ*ansAB::ermc* Δ*ansZ::kan* Δ*azlBCD::spec* Δ*aimA::phleo* Δ*bcaP::cat*	GP4266 --> GP4245
GP4269	*trpC2* Δ*cdaA::cat* Δ*cdaS::ermC* Δ*disA::tet ktrD* $_{fs}$ *(pos 311 –CAGTCGG)*	Suppressor of GP2222 (15 mM L-Asn)
GP4270	*trpC2* Δ*cdaA::cat* Δ*cdaS::ermC* Δ*disA::tet ktrD* $_{fs}$ *(pos 24 –T)*	Suppressor of GP2222 (15 mM L-Asn)

7.3. Plasmids

7.3.1. Plasmids used in this work

Name	Description	Construction/ Reference
pAC5	Construction of translational *lacZ* fusions in *B. subtilis*, integrates into the *amyE* site	(Martin-Verstraete et al., 1992)
pAC7	Construction of translational *lacZ* fusions in *B. subtilis*, integrates into the *amyE* site	(Weinrauch et al., 1991)
pGP1460	Constitutive expression of C-terminally Strep-tagged proteins in *B. subtilis*, integrates into the *ganA* site	(Mehne et al., 2013)
pDG148	Plasmid for amplification of the phleo-resistance gene for LFH-PCR	(Guérout-Fleury et al., 1995)
pDG646	Plasmid for amplification of the ermC-resistance gene for LFH-PCR	(Guérout-Fleury et al., 1995)
pDG780	Plasmid for amplification of the kan-resistance gene for LFH-PCR	(Guérout-Fleury et al., 1995)
pDG1513	Plasmid for amplification of the tet-resistance gene for LFH-PCR	(Guérout-Fleury et al., 1995)
pDG1726	Plasmid for amplification of the spec-resistance gene for LFH-PCR	(Guérout-Fleury et al., 1995)
pGEM-cm	Plasmid for amplification of the cat-resistance gene for LFH-PCR	(Guérout-Fleury et al., 1995)

7.3.2. Plasmids constructed in this work

Plasmid	Vector	Construction
pGP3807	pAC7/ BamHI + EcoRI	PCR product *yrdF* promoter JM218/JM219 (BamHI + EcoRI)
pGP3808	pAC7/ BamHI + EcoRI	PCR-product *azlB* promoter JM220/JM221 (BamHI + EcoRI)
pGP3811	pGP1460/ BamHI + PstI	PCR product *azlC* JM269/JM271 (BamHI + PstI)
pGP3812	pGP1460/ BamHI + PstI	PCR product *azlCD* JM269/JM270 (BamHI + PstI)

7.4. Oligonucleotides

7.4.1. Oligonucleotides designed for this work

Name	Sequence 5'→3', restriction sites are underlined, extra bp are in bold	Purpose
KG28	ATGGCTTGGACCCGTTATTGGGG	fwd. primer upstream LFH for deletion of *ansAB*
KG29	CCTATCACCTCAAATGGTTCGCTGGAGCCAGCCCATTTTC CCCTTC	rev. primer upstream LFH for deletion of *ansAB*
KG30	CCGAGCGCCTACGAGGAATTTGTATCG CGGCGCTGATCATCTTGTTGATG	fwd. primer downstream LFH for deletion of *ansAB*
KG31	AAGTCGGCACAACGCCTCCGG	rev. primer downstream LFH for deletion of *ansAB*
KG32	GCGCATTCGTTGGGGAAAATCGG	fwd. primer sequencing *ansAB* deletion
KG33	GCCGGCAGTTCCGACTGTTCC	rev. primer sequencing *ansAB* deletion
JN329	CCTATCACCTCAAATGGTTCGCTG GCAATATAGATGACAGGTCTAACTCGT	rev. primer upstream LFH for deletion of *disA*
JN330	CCGTCACTGCATGTTTTATCTGAAA	fwd. primer upstream LFH for deletion of *disA*

JN331	CCGAGCGCCTACGAGGAATTTGTATCG GGATTAAAACGCCTGCAAGAGAAG	fwd. primer downstream LFH for deletion of *disA*
JN332	CTTCTGAAGCTCGCACACTTTATTT	rev. primer downstream LFH for deletion of *disA*
JN333	CCGCTCAATTATCAGGCTCATCA	fwd. primer sequencing *disA* deletion
JN334	CCCTGATTATGCTCTTTCCCGT	rev. primer sequencing *disA* deletion
DR141	AAACAGCCGGGAAAGAGAAATGTTTGTG	fwd. primer upstream LFH for deletion of *aimA*
DR142	CCTATCACCTCAAATGGTTCGCTGCAATTGATTCATATCG TAAACACCTTTCATGTATGA	rev. primer upstream LFH for deletion of *aimA*
DR143	CGAGCGCCTACGAGGAATTTGTATCGGAATAAAATATAG AAGAAAACCTTGCGATAGTTGTC	fwd. primer downstream LFH for deletion of *aimA*
DR144	CACGCCGTTTACATCCGTTTTTTATTATGAAAG	rev. primer downstream LFH for deletion of *aimA*
DR145	GGCCTATTGTCGCCATTTCTTACTT	fwd. primer, sequencing *aimA* deletion
DR146	ATTCTTGCCATCGTCTTCTTGGCT	rev. primer, sequencing *aimA* deletion
JM213	CCTATCACCTCAAATGGTTCGCTGTTCGTCAAGCATAATA CCCATTTCAC	fwd. primer upstream LFH for deletion of *azlB*
JM214	CAAAGATTGCGATTTCCACCTTATTAC	rev. primer upstream LFH for deletion of *azlB*
JM215	AATATTTTACTGGATGAATTGTTTTAGTAATGGTTTTGGT GCAATTGGTTTTTTAA	rev. primer downstream LFH for deletion of *azlB*
JM216	CCCAATAAGATTGAACCATAAGATCCAAAA	fwd. primer downstream LFH for deletion of *azlB*
JM217	CCGAGCGCCTACGAGGAATTTGTATCGCTGTCAGTGTGC CAATTGATGAAA	rev. primer downstream LFH for deletion of *azlB*
JM218	GGATCCCGCATTAGAAACTATGCCCTCCATTATTTTATCT	*yrdF* promoter region *lacZ* fusion for pAC7
JM219	GAATTCTTTTTAGGGGATATGTGATTTTCAAACATAT	*yrdF* promoter region *lacZ* fusion for pAC7

JM220	GGATCCCGCATTTCACAACCCCCAATACAACTTA	*azlB* promoter region *lacZ* fusion for pAC7
JM221	GAATTCACTTTCTGTTTTGCGAATTTTAACAATTTTC	*azlB* promoter region *lacZ* fusion for pAC7
JM241	CGAGCCAGTCCCTATCACCTCAAATGGTTCGCTGGCAGTA GTGAGAAAAACCTGTAACAA	rev. primer upstream LFH for deletion of *gltR*
JM242	CCACCTATAGCTTCTATAATCATATAACCTGT	fwd. primer upstream LFH for deletion of *gltR*
JM243	CCGAGCGCCTACGAGGAATTTGTATCGAACGCATATGAG GGATCATTTTCAG	fwd. primer downstream LFH for deletion of *gltR*
JM244	CCTATTATTTTGGATCTTATGGTTCAATCTTATTG	rev. primer downstream LFH for deletion of *gltR*
JM245	CAAGGAGCTTCTACCAAAAAACAGC	fwd. primer sequencing *gltR* deletion
JM246	GAATGTCTTATTGTGATTTGCCTTTTTTT	rev. primer sequencing *gltR* deletion
JM253	CTAATACGACTCACTATAGGGAGA TTTCTCCAATGGTTTTCTCAGAACAG	fwd. primer for in vitro transcription of *azlC,* incl. T7 prom. region
JM254	AAAATAAAGAATCGTTACATTTAAGCTCTCCT	rev. primer for in vitro transcription of *azlC,* incl. T7 prom. region
JM255	CTAATACGACTCACTATAGGGAGA ACATAGGAAGAATCTGAGTGAAAATACG	fwd. primer for in vitro transcription of *brnQ,* incl. T7 prom. region
JM256	AAAAAAAGTGTCTGCTTCCTATATCATCAT	rev. primer for in vitro transcription of *brnQ,* incl. T7 prom. region
JM257	CCTATCACCTCAAATGGTTCGCTGAGGAGAGCTTAAATGT AACGATTCTTTATT	fwd. primer upstream LFH for deletion of *azlCD*
JM264	TGACAGGGTGGGTGGATTTG	rev. primer upstream LFH for deletion of *azlCD*
JM269	AAA GGATCC AAAGGAGGAAACAATC TTGAATAAAAATAAAGAATCGTTACATTTAAGCTC	rev. primer amplification of *azlC* and *azlCD* for pGP1460

JM270	AAA CTGCAG TTAAAAAACCAATTGCACCAAAACCATATAAA	rev. primer amplification of *azlCD* for pGP1460
JM271	AAA CTGCAG TCATACGGAAACCTCAGCTTTCTC	fwd. primer, amplification of *azlC* for pGP1460
JM359	CGAGCCAGTCCCTATCACCTCAAATGGTTCGCTG CTGTTGTCAATGAAGAACCGTCTAAA	rev. primer upstream LFH for deletion of *hutH*
JM360	CTTGCATATCCTCTCCTTCATAGTTCA	fwd. primer upstream LFH for deletion of *hutH*
JM361	CCGAGCGCCTACGAGGAATTTGTATCG GACATTGAACGCTTGACTGACTG	fwd. primer downstream LFH for deletion of *hutH*
JM362	TTGTTGCCGTAGTCAAATACAATAGAG	rev. primer downstream LFH for deletion of *hutH*
JM363	CCTCTCTTTTTTATTAAATTTTAAACGCTTACATT	fwd. primer sequencing *hutH* deletion
JM364	GGGAACAGCTCTTTTAATAACGCA	rev. primer sequencing *hutH* deletion
JM365	GGTTACCCTGCTTCTACTAATCTTGAT	fwd. sequencing primer *disA* deletion
JM366	ATAACCGAGGAAGAAAGCCAAAAATAC	rev. sequencing primer *disA* deletion
JM449	CCTATCACCTCAAATGGTTCGCTG GAAGAACAAGAGAAACCGGATGATC	rev. primer upstream LFH for deletion of *bcaP*
JM450	TGACCATTGTTACTCATGAATTCAGAAA	fwd. primer upstream LFH for deletion of *bcaP*
JM451	CCGAGCGCCTACGAGGAATTTGTATCG CTATTTCCTGTATTCAAGAAAGCATTCAAAA	fwd. primer downstream LFH for deletion of *bcaP*
JM452	GCTGAAAACCGAATGAGAAAACC	rev. primer downstream LFH for deletion of *bcaP*
JM453	GAGCTGGTTTTTTGTGTGGTGAA	fwd. primer sequencing *bcaP* deletion
JM454	AAAATGCTGAGGTTAAGGCTGC	rev. primer sequencing *bcaP* deletion

7.4.2. Additional oligonucleotides used in this work

Name	Sequence 5'→3', restriction sites are underlined, extra bp are in bold	Purpose
cat-rev (kan)	CGATACAAATTCCTCGTAGGCGCTCGGCCAGCGTGGACCGGCG AGGCTAGTTACCC	amplification of the *cat* resistance gene for LFH PCR
cat-fwd (kan)	CAGCGAACCATTTGAGGTGATAGGCGGCAATAGTTACCCTTATT ATCAAG	amplification of the *cat* resistance gene for LFH PCR
cat-rev (kan) w/o terminator	CGATACAAATTCCTCGTAGGCGCTCGGTTATAAAAGCCAGTCAT TAGGCCTATC	amplification of the *cat* resistance gene for LFH PCR
cat check fwd	CTAATGTCACTAACCTGCCC	sequencing out of *cat* resistance gene
cat check rev	GTCTGCTTTCTTCATTAGAATCAATCC	sequencing out of *cat* resistance gene
kan fwd	CAGCGAACCATTTGAGGTGATAGG	amplification of the *kan* resistance gene for LFH PCR
kan rev	CGATACAAATTCCTCGTAGGCGCTCGG	amplification of the *kan* resistance gene for LFH PCR
kan-rev w/o terminator	TTACTAAAACAATTCATCCAGTAAAATAT	amplification of the *kan* resistance gene for LFH PCR
kan check fwd	CATCCGCAACTGTCCATACTCTG	sequencing out of *kan* resistance gene
kan check rev	CTGCCTCCTCATCCTCTTCATCC	sequencing out of *kan* resistance gene
spec-fwd (kan)	CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACG TAACGTGACTGGCAAGAG	amplification of the *spec* resistance gene for LFH PCR

spec-rev (kan)	CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGTTTATTGTTTTCTAAAATCTG	amplification of the *spec* resistance gene for LFH PCR
JG69	CGATACAAATTCCTCGTAGGCGCTCGGGTTTCCACCATTTTTTCAATTTTTTTATAATTTTTTTAATCTG	amplification of the *spec* resistance gene for LFH PCR
spec check fwd	GTTATCTTGGAGAGAATATTGAATGGAC	sequencing out of *spec* resistance gene
spec check rev	CGTATGTATTCAAATATATCCTCCTCAC	sequencing out of *spec* resistance gene
tc-fwd (kan)	CGATACAAATTCCTCGTAGGCGCTCGGGAACTCTCTCCCAAAGTTGATCCC	amplification of the *tet* resistance gene for LFH PCR
tc-rev (kan) w/o terminator	CGATACAAATTCCTCGTAGGCGCTCGGCGATTTAGAAATCCCTTTGAGAATGTTT	amplification of the *tet* resistance gene for LFH PCR
tc check rev	CATCGGTCATAAAATCCGTAATGC	sequencing out of *tet* resistance gene
tc check fwd	CGGCTACATTGGTGGGATACTTGTTG	sequencing out of *tet* resistance gene
mls check fwd	CCTTAAAACATGCAGGAATTGACG	sequencing out of *ermC* resistance gene
mls check rev	GTTTTGGTCGTAGAGCACACGG	sequencing out of *ermC* resistance gene
mls-fwd (kan)	CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACTCTGGCAACCCTC	amplification of the *ermC* resistance gene for LFH PCR
mls-rev (kan)	CGATACAAATTCCTCGTAGGCGCTCGGGCCGACTGCGCAAAAGACATAATCG	amplification of the *ermC* resistance gene for LFH PCR

CZ126	CAGCGAACCATTTGAGGTGATAGGGAACGATGACCTCTAATAATTG	amplification of the *phleo* resistance gene for LFH PCR
CZ127	CGATACAAATTCCTCGTAGGCGCTCGGGTAGTATTTTTTGAGAAGATCAC	amplification of the *phleo* resistance gene for LFH PCR
CZ128	CCAAAGTGAAACCTAGTTTATC	sequencing out of *phleo* resistance gene
CZ129	CGAGACTTTGCAGTAATTGATC	sequencing out of *phleo* resistance gene
LK109	TCATACGGAAACCTCAGCTTTCTCCAATG	fwd. primer downstream LFH for deletion of *azlB*
LK104	ATAGAAGTTTTACATGATGATTTAAAAGATAAACTAGACTTCC	rev. primer upstream LFH for deletion of *azlCD*