Resistance to serine in *Bacillus subtilis*: identification of the serine transporter YbeC and of a metabolic network that links serine and threonine metabolism

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Summary

The Gram-positive bacterium *Bacillus subtilis* uses serine not only as a building block for proteins but also as an important precursor in many anabolic reactions. Moreover, a lack of serine results in the initiation of biofilm formation. However, excess serine inhibits the growth of *B. subtilis*. To unravel the underlying mechanisms, we isolated suppressor mutants that can tolerate toxic serine concentrations by three targeted and non-targeted genome-wide screens. All screens as well as genetic complementation in *Escherichia coli* identified the so far uncharacterized permease YbeC as the major serine transporter of *B. subtilis*. In addition to YbeC, the threonine transporters BcaP and YbxG make minor contributions to serine uptake. A strain lacking these three transporters was able to tolerate 100 mM serine whereas the wild type strain was already inhibited by 1 mM of the amino acid. The screen for serine-resistant mutants also identified mutations that result in increased serine degradation and in increased expression of threonine biosynthetic enzymes suggesting that serine toxicity results from interference with threonine biosynthesis.

Introduction

As building block of proteins, amino acids are central to the physiology of any living cell. In addition to their role as substrates in protein biosynthesis, they can be used as carbon and nitrogen sources. Moreover, some amino acids are also required for bacterial cell wall biosynthesis and as osmoprotectants. Accordingly, the acquisition of amino acids is an essential task of all cells. This can be achieved by the direct uptake of amino acids present in the growth medium, by the uptake and intracellular degradation of peptides and by de novo biosynthesis. Many bacteria such as the model organisms *Escherichia coli* and *Bacillus subtilis* are capable of synthesizing all amino acids whereas others such as the minimal bacteria of the genus *Mycoplasma* completely depend on the uptake of amino acids.

While amino acids are essential for the cells, increased concentrations of some amino acids such as glutamate, threonine or serine can be harmful (Lamb and Bott, 1979a; Lamb and Bott, 1979b; Lachowicz et al., 1996; Belitsky and Sonenshein, 1998; Ogawa et al., 1998; Commichau et al., 2008; Belitsky, 2015; Mundhada et al., 2017). Therefore, the homeostasis of the amino acids must be tightly controlled to adjust the intracellular levels of each amino acid to the actual need of the cell. This requires balanced activities of systems for amino acid acquisition and degradation. For the Gram-positive model bacterium *B. subtilis*, amino acid metabolism is one of the few functions in core metabolism that have not yet been completely elucidated. This is the case both for the biosynthetic pathways and for amino acid transport.

The genome of *B. subtilis* encodes 47 known and predicted amino acid transporters (Zhu and Stülke, 2018). For 19 of these transporters, substrates have been identified, and for four additional transporters, tentative substrates can be assigned based on mutant properties (for YbxG; Commichau et al., 2015) and on the assignment to particular regulons (AlsT, YvbW and YysH; Randazzo et al., 2017; Wels et al., 2008; Rodionov et al., 2003). No functional assignment can so far be made for 11 potential transporters. It is important to note that some proteins that are members of typical amino
acid transporter families do actually transport other substrates, such as the recently described potassium transporter KimA (Gundlach et al., 2017). A complete overview on the known and potential amino acid transporters of *B. subtilis* can be found in Table S1 (see also http://subtiwiki.uni-goettingen.de/v3/category/view/SW%201.2, Zhu and Stülke, 2018). Importantly, no transporters have so far been identified or proposed for alanine, glycine, serine, asparagine, and the aromatic amino acids phenylalanine and tyrosine. The identification of new amino acid transporters is hampered by two peculiarities: For one amino acid, there are often multiple transporters, as has been shown for arginine, proline, or the branched-chain amino acids (Calogero et al., 1994; Gardan et al., 1995; Sekowska et al., 2001; Zaprasis et al., 2014; Belitsky, 2015). On the other hand, many permeases have a relatively weak substrate specificity, i.e. they are able to transport multiple amino acids, as shown for BcaP or GltT (Belitsky, 2015; Zaprasis et al., 2015; Wicke et al., 2019).

We are interested in the identification of the functions that are required to sustain the life of a minimal cell and in the corresponding set of genes and proteins. In an analysis of the genome of *B. subtilis*, amino acid transporters were proposed to be kept in a minimal genome rather than biosynthetic genes, since this would require fewer genes (Reuß et al., 2016). However, as indicated above, no transporters have been identified for several amino acids. Accordingly, biosynthetic pathways were included for those amino acids. A minimal organism capable of transporting amino acids but not to produce them is expected to be viable on complex media but would be unable to grow on minimal medium in the absence of added amino acids. As minimal bacterial strains have a huge potential for biotechnological applications (Suárez et al., 2019), the ability to produce amino acids may be important for growth on cheap minimal salts substrates.

Serine is an important amino acid because this molecule is not only a building block for protein synthesis but also a precursor of nucleotides, phospholipids, redox molecules and other amino acids. In addition, decreased level of intracellular serine can be a signal for initiation of biofilm formation in *B. subtilis* (Subramanian et al., 2013), suggesting that regulation of serine homeostasis is very important. However, the metabolism of serine is not yet completely understood in *B. subtilis*. For this amino acid, no transporter has been identified, and the knowledge of biosynthetic pathways has remained limited until recently. Indeed, the serine biosynthesis pathway has been completed just recently in the frame of a genome-scale deletion study by the identification of the SerB phosphoserine phosphatase that catalyses the last step of the pathway (Koo et al., 2017) (see Fig. 1 for an overview on serine metabolism in *B. subtilis*). Moreover, the reasons for serine toxicity have remained enigmatic. In *E. coli*, it has been suggested that serine binds and inactivates the bifunctional enzyme aspartate kinase/homoserine dehydrogenase (ThrA) (Mundhada et al., 2017) thus interfering with threonine biosynthesis. In addition, serine catabolism can give rise to toxic intermediates, the highly reactive and therefore toxic β-hydroxypyruvate as product.

![Fig. 1. Serine and threonine metabolic pathways in *B. subtilis*. The model shows the relevant transporters, the biosynthesis of threonine, and its role as a precursor for isoleucine biosynthesis as well as the pathways for serine biosynthesis and degradation. [Color figure can be viewed at wileyonlinelibrary.com]](https://example.com/fig1.png)

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of the transamination reaction, and α-aminoacrylate, which is formed as byproduct of serine deamination (Blatt et al., 1966; Seebeck and Szostak, 2006, see de Lorenzo et al., 2015 for review).

In this work, we have taken advantage of the serine toxicity phenotype of B. subtilis to devise reverse and forward genetic screens to identify serine transporters. Our analysis identified YbeC as the major serine transporter, and clarified the roles of threonine transporters in uptake of serine. Using a suppressor screen, we also isolated metabolic mutants that circumvent serine toxicity. These mutants exhibit either more efficient serine degradation or overexpression of genes for the threonine and isoleucine biosynthetic pathways suggesting that one or more enzymes in this pathway are inhibited by serine.

Results
Overview of the genetic approaches used in this work
All of our screens and selections were based on the fact that addition of serine to minimal medium is toxic for B. subtilis 168, whereas the addition of serine to complex LB medium did not interfere with growth of the bacteria. Moreover, the addition of specific amino acids such as threonine to minimal medium also overcomes serine toxicity (Vandeyar and Zahler, 1986; Lachowicz et al., 1996). These observations suggest that intracellular serine interferes with amino acid metabolism. We might therefore expect that strains would become resistant to serine toxicity either by eliminating the major serine transporter or by altering amino acid metabolism of genes related to serine toxicity. To identify these genes, we used the following approaches:

A targeted screen for transporters. For this purpose, we chose 12 candidate transporters that met two criteria: First, these transporters have been poorly studied in B. subtilis, and second, they are expressed during vegetative growth. These transporters are AapA, AlsT, MtrA, SteT, YbeC, YbgF, YdgF, YecA, YodF and YtnA. Mutants for the corresponding genes (see Table S2) were constructed and analysed for the ability to grow in the presence of 244 μM L-serine. While all strains were capable of growing on minimal medium in the absence of serine, only the ybeC mutant strain GP1886 was able to grow in the presence of serine, suggesting that YbeC might act as serine transporter.

A suppressor screen aimed at identifying mutants altered in related amino acid metabolism. We selected for loss of serine toxicity in the wild type strain and in a ΔserA mutant that is auxotrophic for serine strain and depends on the uptake of serine for growth. Of eight studied suppressor strains, four were transporter (ybeC) mutants and the remaining strains exhibited genetic lesions related to amino acid metabolism. Interestingly, the ybeC mutation was also found in the serA mutant as a suppressor, indicating that serine can be transported in ybeC mutant.

A screen of the entire B. subtilis deletion library for loss of serine toxicity. In order to make sure that the screens described above were exhaustive, we also made use of the deletion library that encompasses all non-essential genes of B. subtilis (Koo et al., 2017). In this library, each reading frame is replaced by an antibiotic cassette with a relatively strong outwardly facing promoter, so resistance to serine toxicity could result from gene deletion or from overexpression of downstream genes. To distinguish between these possibilities, we removed the antibiotic cassette and retested the phenotype. If the phenotype was retained following removal of the antibiotic cassette, then the phenotype was caused by gene deletion; if not it was likely due to overexpression of downstream genes. This screen identified both the transporter, YbeC, and genetic lesions related amino acid metabolism (Table 1).

Table 1. Serine-resistant mutants identified from the genome-wide screen.

| Strain⁴ | Genetic context | Serine resistance | Determinant for serine resistance |
|---------|-----------------|------------------|----------------------------------|
| ΔybeC::erm | ybeC>, <glpQ < glpT | Yes | Deletion of ybeC |
| ΔybeC::lox72 | ybeC>, <glpQ < glpT | Yes | Inhibition of ybeC expression |
| ΔglpC::erm | ybeC>, <glpQ < glpT | Yes | Overexpression of sdaAB-AA |
| ΔglpC::lox72 | ybeC>, <glpQ < glpT | Yes | Overexpression of sdaAB-AA |
| ΔyloU::erm | yloU > yloV, sdaAB > sdaAA > recG | No | Deletion of thrR |
| ΔyloU::lox72 | yloU > yloV, sdaAB > sdaAA > recG | No | Overexpression of hom-thrCB |
| ΔyloV::erm | yloV > yloU, sdaAB > sdaAA > recG | No | Overexpression of hom-thrCB |
| ΔyloV::lox72 | yloV > yloU, sdaAB > sdaAA > recG | No | Overexpression of hom-thrCB |

*lox72 indicates the scar resulting from looping out of erythromycin-resistant cassette.
The genes highlighted in bold are directly responsible for the resistance to serine.

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Identification of a permease that confers sensitivity to L-serine

Both our targeted screen of the 13 expressed, uncharacterized transporters and our screen of the B. subtilis deletion library identified only a single putative transporter, YbeC, the loss of which conferred resistance to serine. Supporting the importance of YbeC, 50% of the mutants from the suppressor screen (Selection #2) had mutations targeting ybeC. Additionally, the glpQ mutant (BKE02130), a strain from the whole-genome screen (Selection #3) that suppressed serine toxicity due to overexpression, likely generates an abundant ybeC antisense RNA (Table 1). The net effect of antisense expression is to decrease ybeC expression, explaining its serine-resistance phenotype.

To test whether the ybeC mutant is also resistant to higher concentrations of serine, we cultivated the mutant GP1886 at increasing serine concentrations (up to 100 mM), andrecorded growth of the bacteria. While the wild type strain was unable to grow at concentrations exceeding 244 μM, the ybeC mutant was able to tolerate as much as 11 mM serine (see Table 2). In addition to serine, the anti-metabolite serine hydroxamate also inhibits growth of B. subtilis. To test whether loss of YbeC allows growth in the presence of this serine analogue, we cultivated the wild type strain 168 and the ybeC mutant GP1886 in the presence of DL-serine hydroxamate (1 mg ml⁻¹). As shown in Fig. 2A, the wild type was sensitive to this molecule whereas the ybeC mutant was somewhat more resistant. Thus, loss of YbeC confers resistance to both serine and its toxic analogue serine hydroxamate, suggesting that the protein is a serine transporter.

Isolation and initial characterization of mutants that are able to grow in the presence of serine

The targeted analysis of potential amino acid transporters identified YbeC as the only candidate serine transporter. In an attempt to identify more genes involved in serine toxicity, we cultivated the B. subtilis wild type strain 168 in the presence of different concentrations of serine. Moreover, we used the serine auxotrophic serA mutant that depends on the uptake of serine for growth, and the ybeC mutant that already tolerates up to 11 mM of serine (see above). In total, we isolated eight mutants that exhibited increased resistance to serine in five distinct selection experiments. One mutant for each selection was subjected to whole-genome sequencing to identify the underlying mutations. In one of the mutants (GP2324, isolated from the wild type 168 at 1 mM serine), a ybeC mutation was detected. Thus, we tested the remaining mutants for the presence of mutations in ybeC. Strikingly, four out of the eight mutants had acquired mutations in ybeC. These mutations resulted in the production of truncated and therefore possibly inactive YbeC proteins or in

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Table 2. The resistance of selected B. subtilis mutants towards serine.

| Strain | Relevant genotype | Tolerated serine concentration (mM)* |
|--------|------------------|-------------------------------------|
| 168    | Wild type        | <1                                  |
| GP2786 | ΔybeC            | 11                                  |
| BKE09460 | ΔbcaP          | 2                                   |
| GP2396 | ΔybxG            | 1.5                                 |
| GP2949 | ΔybeC ΔbcaP      | 40                                  |
| GP2951 | ΔybeC ΔybxG      | 25                                  |
| GP2952 | ΔbcaP ΔybxG      | 4                                   |
| GP2950 | ΔybeC ΔbcaP ΔybxG | 100                                |

*The tolerated serine concentrations were determined by cultivating the strains in liquid C-Glc minimal medium in the presence of different serine concentrations. Note that the results obtained with plates and liquid medium can differ slightly.
an in-frame deletion of 236 amino acids (in GP3050). The identification of multiple suppressor mutants affecting YbeC strongly supports the crucial role of YbeC in the resistance to serine.

Of the serine-resistant strains whose phenotype was not caused by a ybeC mutation, two strains derived from the wild type strain 168 had a duplication of about 16 kb yokD-thyB chromosomal region. Interestingly, this region contains the ilvA gene encoding threonine dehydratase involved in the biosynthesis of isoleucine from threonine. The remaining three mutants (derived from the serA mutant, and the ybeC mutant at 10 and 17 mM serine, see Table S2 for details) had mutations affecting the repressor for the threonine biosynthetic genes, thrR (Rosenberg et al., 2016), and mutations in the regulatory regions of the sdaAB and hom promoter regions respectively (Table S2). All these genes are involved in serine and threonine metabolism suggesting a close relation between the metabolic pathways for these similar amino acids (see below for further analyses).

YbeC is a serine transporter

All three targeted and unbiased analyses of serine-resistance mutants identified YbeC as the main player. YbeC is similar to known amino acid transporters, is classified as a member of the amino acid-polyamine-organocation superfamily (see Table S1), and the ybeC mutant had the phenotypes expected for a major serine transporter. To test this idea, we made use of an E. coli mutant that lacks the major serine transporter SstT. This strain is less sensitive to growth inhibition by serine (Ogawa et al., 1997; Ogawa et al., 1998). We cloned the ybeC gene into the expression vector pWH844 and used the resulting plasmid pGP2987 to transform the sstT mutant JW3060 (Baba et al., 2006). Indeed, the expression of plasmid-borne ybeC in E. coli JW3060 restored serine toxicity (Fig. 2B). Taken together, both the genetic characterization and the functional complementation of an E. coli mutant lacking a serine transporter demonstrate that YbeC is indeed a transporter for serine.

The ybeC gene forms a monocistronic transcription unit (Nicolas et al., 2012). To study the activity of the ybeC promoter and its possible regulation by serine, a 257 bp region (222 bp upstream of the ATG translational start codon, and 35 bp of the ybeC coding region) was fused to a promoterless lacZ gene. The resulting strain, GP2965, was cultivated in minimal in the presence and absence of serine as well as in complex (LB) medium. With very similar β-Galactosidase activities (144 ± 2, 132 ± 8 and 135 ± 10 units mg⁻¹ of protein respectively), this fusion was similarly expressed irrespective of the presence of serine in the medium thus indicating constitutive expression of ybeC.

Serine and threonine share overlapping transporters

Threonine transporters contribute to serine uptake. The identification of viable serA ybeC mutants in the suppressor screen (Screen #2 above) suggested either that the mutant YbeC proteins retained some transport activity or that YbeC is not the only transporter for serine. To resolve this issue, we deleted the ybeC gene in the serA mutant, which is auxotrophic for serine. The resulting double mutant GP2941 depends on serine uptake for viability. Analysis of growth of these mutants demonstrated that both the serA mutant and the serA ybeC double mutant were unable to grow in the absence of serine (C-glucose medium). In contrast, the strains lacking the ybeC gene were able to grow in minimal medium supplemented with serine (see Fig. 3A). Thus, the ∆ybeC mutant is still able to transport serine.

Serine and threonine are chemically similar to each other and the E. coli SstT transporter is capable of transporting both serine and threonine (Kim et al., 2002). Therefore, we considered the possibility that threonine transporters might contribute to serine uptake in B. subtilis, and vice versa. Based on the analysis of growth inhibition by threonine and its analogue 4-hydroxythreonine, the BcaP and YbxG permeases have been identified as tentative threonine transporters in B. subtilis (see Table S1, Belitsky, 2015; Commichau et al., 2015). To test the possible role of these permeases in serine transport, we used single, double and triple mutants lacking ybeC, bcaP and ybxG respectively. The resulting strains were assayed for their resistance towards serine. As shown in Fig. 3B, the single bcaP and ybxG deletions conferred only a weak resistance to growth inhibition by serine, whereas the loss of ybeC resulted in a substantial resistance (see also Table 2). This observation confirms that YbeC is the main transporter for serine in B. subtilis.

The double mutants lacking YbeC and one of the threonine transporters exhibited a substantial increase in resistance to serine indicating that both threonine permeases contribute to serine uptake (see Table 2). In contrast, the bcaP ybxG double mutant was much more sensitive to serine than the ybeC mutant. This observation supports the conclusion that YbeC is the major serine permease. The analysis of double mutants lacking YbeC and either YbxG or BcaP indicates that the loss of BcaP has a higher contribution to serine resistance as compared with the loss of YbxG (Table 2, compare GP2951 and GP2949). This indicates that BcaP may be more active in serine transport than YbxG. The deletion of the three permease-encoding genes in the triple mutant GP2950 resulted in an unprecedented resistance to serine up to 100 mM (Table 2). This finding indicates that these three proteins may be responsible for the majority of serine uptake in B. subtilis. If these proteins were the only serine permeases, one would expect that
an auxotrophic serA mutant lacking the three permeases would not be viable. However, this mutant (GP2955) was still able to grow on minimal medium in the presence of serine (Fig. 3C). Thus, B. subtilis possesses at least one additional permease that is able to transport serine.

Analysis of threonine transport. Our findings demonstrate that the two previously suggested threonine transporters are also active as minor serine permeases. Next, we asked whether YbeC is also capable of transporting threonine. To address this question, we made use of the observation that threonine is toxic for B. subtilis if added in concentrations exceeding 50 μg mL⁻¹ (Lamb and Bott, 1979a; Lamb and Bott, 1979b). In our experimental setup, threonine (10 mM) inhibits growth of B. subtilis 168. Inactivation of the bcaP gene conferred a growth advantage, the bcaP mutant grew in the presence of threonine as well as the wild type strain in the absence of this amino acid. In contrast, the deletions of ybxG or ybeC had only minor effects (Fig. 4A). This observation is supported by the analysis of the double and triple mutants: While all mutants lacking bcaP showed threonine-resistant growth, this was not the case for the ybeC ybxG double mutant GP2952 that still expresses BcaP (Fig. 4B). These observations suggest that BcaP is the main threonine transporter in B. subtilis.

In order to test the presence of additional threonine transporters, and to get further evidence for the relative roles of BcaP, YbxG and YbeC in threonine uptake, we deleted the thrC gene in the wild type 168 and in relevant transporter mutants. The thrC gene codes for threonine synthase which catalyses the final step in threonine biosynthesis. As expected, the thrC mutant was auxotrophic for threonine (data not shown). The deletion of bcaP alone or in combination with ybeC resulted in improved growth both at 0.04 and 4 mM threonine as compared with the single thrC mutant (see Fig. 5). The combination of the thrC, ybeC and ybxG mutations had no effect as compared with the single thrC deletion supporting the idea that YbeC and YbxG play only very minor roles in threonine uptake. However, the simultaneous deletion of bcaP and ybxG in the thrC mutant resulted in severely reduced growth at 0.04 mM threonine (Fig. 5A). The additional deletion of ybeC had only a minor, if any impact. These findings suggest that BcaP and YbxG act as threonine transporters. Importantly, the thrC mutant lacking

Fig. 3. The contribution of threonine transporters to serine uptake. Cells of the indicated strains were grown in C-Glc minimal medium to an OD₆₀₀ of 1.0 and serial dilutions (10-fold) were prepared. These samples were plated on C-Glc minimal plates containing no serine or 1 mM serine. The plates were incubated at 37°C for 48 h.

A. Combination of the ybeC deletion with the deletion of the serA gene encoding phosphoglycerate dehydrogenase. The growth of the single deletion mutants of ybeC (GP1886) and serA (GP2392) was compared with the growth of the combined deletion strain of ybeC and serA (GP2941).

B. The resistance of the threonine transporter deletion strains to serine. The bcaP (BKE09460) and ybxG deletion strains (GP2396) are compared with the wild type strain 168 and the ybeC deletion strain (GP1886). C. Combination of the serA deletion with the deletion strain if bcaP, ybeC and ybxG. The growth of the wild type strain 168 was compared with GP2392 (serA), GP2955 (serA bcaP ybeC ybxG) and GP2950 (bcaP ybeC ybxG).
BcaP and YbxG (and YbeC) is still able to grow in the presence of threonine suggesting the existence of at least one additional threonine transporter (Fig. 5A and B). Taken together, these results indicate that BcaP is the major threonine transporter in *B. subtilis*, whereas YbxG has a minor threonine permease activity. Our data do not support the annotation of YbeC as a threonine transporter. Moreover, BcaP and YbxG have overlapping activity for both serine and threonine (see Fig. 1).

**Putting serine toxicity in its metabolic context**

As mentioned above, the addition of serine to minimal medium is toxic, whereas its addition to complex LB medium did not interfere with growth of *B. subtilis* 168 (data not shown). The inactivation of the *ybeC* gene to prevent serine uptake or the addition of several individual amino acids such as threonine overcomes serine toxicity (Vandeyar and Zahler, 1986; Lachowicz et al., 1996). These observations suggest that intracellular serine interferes with amino acid metabolism. Mutants from the suppressor screen (Selection #2) and from the genome wide screen (Selection #3) shed light on the origins of serine toxicity.

The role of serine deaminase in overcoming serine toxicity.

The *sdaAB-sdaAA* operon encodes the two subunits of serine deaminase, which catalyses the degradation of serine to pyruvate and ammonia (Chen et al., 2012). Both the suppressor screen (Selection #2) and the whole-genome screen (Selection #3) identified overexpression of *sdaAB-sdaAA* as relieving serine toxicity. In the suppressor screen, strain GP2971 had a mutation 70 bp upstream of the start of the *sdaAB* coding sequence suggesting that it might affect expression of the operon. Indeed, a promoter has been identified in the 139 bp intergenic region between the *yloV* and *sdaAB* genes.
To test this hypothesis, we fused the 166 bp wild type and mutant regions that contain the complete yloV-sdaAB intergenic region, and thus the sdaAB promoter, to a promoterless lacZ gene, and compared the gene expression driven by these promoters. The strains carrying the lacZ fusions integrated into the amyE gene were cultivated in minimal medium, and their β-Galactosidase activities were determined. For the wild type promoter, we detected 7.4 ± 2.2 units of β-Galactosidase per mg of protein. This corresponds to a very weak promoter activity (Schilling et al., 2007). Expression of the lacZ gene from the mutant promoter resulted in 370 ± 48 units of β-Galactosidase per mg of protein. Thus, the mutation resulted in a 50-fold increase of promoter activity. A closer inspection of the sequence around the mutation suggests that a TTGCCA sequence had been altered to the perfect –35 sequence, TTGACA. It is tempting to speculate that this perfect –35 region is responsible for the higher expression of the sdaAB-sdaAA operon and thus for higher intracellular levels of serine deaminase in the mutant. This conclusion is strongly supported by two mutants from the whole-genome screen, affected in yloV (BKE15840) and yloU (BKE15830) that suppressed serine toxicity due to overexpression of the sdaAB-sdaAA operon. These strains have their antibiotic cassettes with the strong outwardly facing promoter immediately upstream of sdaAB-sdaAA, indicating that their overexpression suppresses serine toxicity (Table 1). The increased degradation of serine by serine deaminase is likely to be responsible for the protective action observed upon overexpression of this enzyme.

The role of threonine metabolism in serine toxicity. Two different loci related to threonine metabolism were identified in our screens. First, the suppressor screen identified a duplication of the 16 kb yokD-thyB region containing ilvA as relieving serine toxicity. Second, both the whole-genome and suppressor screens (Selection #2, 3) identified overexpression of the hom-thrC-thrB operon as relieving serine toxicity.

The threonine dehydratase IlvA uses threonine in the initial step of isoleucine biosynthesis. We observed a duplication of the approximately 16 kb yokD-thyB genomic region encompassing ilvA in two suppressor strains. This observation implies that IlvA may become limiting in the presence of serine or contribute to scavenging excess serine. If IlvA is inhibited by serine, this could be compensated by overexpression of ilvA gene (due to genomic duplication) or by increased synthesis of ThrC with its moonlighting activity as threonine dehydratase (Skarstedt and Greer, 1973; Rosenberg et al., 2016) (see Fig. 1) which resumes isoleucine synthesis. However, this is unlikely since supplementation of isoleucine does not reduce serine toxicity (data not shown). Thus, it is more likely that B. subtilis IlvA may also have serine dehydratase activity, resulting in deamination of serine as has been shown in Salmonella enterica and E. coli (Borchert and Downs, 2018). To test whether IlvA is a major determinant for serine resistance in these suppressor strains, we overexpressed the ilvA gene in the wild type strain 168 using the expression vector pGP2289 (see Fig. 6). Indeed, ilvA overexpression provided resistance to serine. However, the level of resistance of the overexpressing strain was lower than observed for the original suppressor mutation with the genomic duplication (see Discussion).

Both the whole-genome and suppressor screens identified inactivation of the ThrR repressor and overexpression of one of its target operons, hom-thrC-thrB as relieving serine toxicity. The suppressor screen (Selection #2) identified a mutation in thrR and a mutation upstream of the hom-thrC-thrB operon. The inspection of the mutation in the hom upstream region revealed that this mutation did affect the ThrR binding site (Rosenberg et al., 2016). Moreover, the thrR mutation (deletion of A91) resulted in a frameshift and translation stop after 35 amino acids. This truncation has been observed.
previously in a different context. It results in an inactive ThrR protein (Rosenberg et al., 2016). This suggests that both the thrR and the hom promoter region mutations result in increased expression of the hom-thrC-thrB operon. To test this idea, we tested the activity of the wild type and mutant hom promoters using hom-lacZ fusions. Strains carrying these fusions were grown in minimal medium and their β-Galactosidase activities were assayed. For the wild type promoter, we detected 275 ± 35 units of β-Galactosidase per mg of protein, whereas the mutant promoter resulted in 970 ± 85 units of β-Galactosidase per mg of protein. These values are similar to those determined previously for the wild type hom promoter and for promoter variants that carry mutations in the ThrR binding site (Rosenberg et al., 2016). Thus, these mutations allow an increased expression of the hom-thrC-thrB operon. These findings are supported by the results from the whole genome screen (Selection #3): The screen identified a strain with a thrR deletion and as well as overexpression of the hom-thrC-thrB operon originating from yutH, which is adjacent to the hom-thrC-thrB operon as relieving serine toxicity (Table 1). Taken together, our results suggest that serine might cause defects in threonine and isoleucine biosynthesis. The defects can be overcome by reducing serine uptake, by degradation of serine, or by an adjustment of threonine and isoleucine metabolism.

Discussion
Metabolite toxicity is one of the least understood areas in the field of microbial metabolism. However, toxic metabolites pose major problems if metabolic pathways are assembled for biotechnological applications or when approaching genome minimization (de Lorenzo et al., 2016) indicates that this minimal Bacillus subtilis species have lost the ability to produce amino acids and therefore depend completely on their uptake from the medium. Due to the fast evolution of this group of bacteria, it has so far not been possible to identify amino acid transporters based on sequence similarity. However, the independent life of the artificial genome-reduced organism Mycoplasma mycoides JCVI-syn3.0 (Hutchinson 3rd et al., 2016) indicates that this minimal bacterium possesses a complete set of amino acid transporters. As mentioned above, serine can inhibit bacterial metabolism both by the interference of the amino acid with other enzymatic reactions, and by the high reactivity toxicity of intrinsic products of serine catabolism (de Lorenzo et al., 2015). One might therefore expect the isolation of different classes of suppressor mutants depending on the ability of the strain to synthesize serine or not. However, all our screens suggest that the amino acid serine rather than derived metabolites is the major obstacle for the metabolic network of B. subtilis. The toxicity of serine can not only be mitigated by the loss of the major serine transporter, YbeC. In addition, our screens also identified other ways to cope with increased serine concentrations, i.e. (i) the rapid conversion of serine to other metabolites, mostly pyruvate and (ii) the over-expression of genes involved in the synthesis of

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threonine (the hom-thrC-thrB operon). The serine deaminase complex SdaAA-AB converts serine to pyruvate and ammonia, thus detoxifying excess serine as well as allowing cells to use serine as carbon and nitrogen source. It is therefore not surprising that removal of this enzyme activity was attempted to increase the yield of serine production in *E. coli* (Li et al., 2012). On the other hand, it was reported that serine deaminase deficiency in *E. coli* resulted in abnormal cell division even in lysogeny broth medium (Zhang and Newman, 2008). Serine deaminase can produce toxic α-aminoacrylate, and overexpression of the enzyme might theoretically result in the accumulation of this intermediate. However, α-aminoacrylate can be disposed of by the activity of the *B. subtilis* YabJ protein (Lambrecht et al., 2012). Importantly, the YabJ protein is much more abundant than the serine deaminase (1660 vs. 90 molecules per cell during growth in minimal medium) (Muntel et al., 2014). The high abundance of YabJ as well as the fact that we did not isolate any suppressor mutants with changed metabolism of serine degradation products suggest that the accumulation of toxic degradation products plays only a minor role in serine toxicity in *B. subtilis*.

There are a couple of ways in which increased expression of the hom-thrC-thrB operon could suppress serine toxicity. First, increased levels of threonine biosynthetic enzymes may produce more threonine. As the addition of threonine to serine-containing minimal medium can partially overcome serine toxicity, it is likely that serine addition deprives the cell of threonine, which can be overcome either by increased threonine synthesis or by external supplementation. Second, L-serine toxicity in *E. coli* works by inhibiting both the aspartate kinase and homoserine dehydrogenase activities of the fused enzyme ThrA (Costrejean and Truffa-Bachi, 1977), and may function analogously in *B. subtilis*. Consistent with this idea, we found that supplementation of homoserine restored the growth of wild type *B. subtilis* in the presence of serine (data not shown). Biochemical analysis with purified *B. subtilis* homoserine dehydrogenase would provide clear evidence for this hypothesis. We attempted to purify *B. subtilis* homoserine dehydrogenase from hom overexpressing *E. coli* strain but failed to get active enzyme. It is tempting to speculate that the increased expression of ilvA upon the duplication the yokD-thyB genomic region is the major determinant for serine-resistant phenotype in this suppressor mutant since we observed that overexpression of *ilvA* pheno-\new{coped it, even though only partially (Fig. 6). One explanation for the incomplete effect of IlvA overexpression is that the enzyme not only suppresses serine toxicity but also is itself toxic to cell due to the accumulation of toxic levels of 2-oxobutanoate or 2-aminoacrylate (Borchert and Downs, 2018). Strikingly, the *ilvA* gene is present in two copies in the suppressor strain whereas it is present on multiple plasmid copies and expressed from a strong constitutive promoter in the artificial overexpression system. This may be too much of a good thing!

This study provides novel insights into important aspects of serine metabolism in *B. subtilis* and into its integration into the amino acid acquisition network. This network consists not only of biosynthetic enzymes with overlapping activities but also of the transporters that are often promiscuous and transport multiple amino acids. Our work provides a starting point for further analysis of the complex and interlocking set of proteins that carry out amino acid transport in *B. subtilis*.

**Methods**

**Bacterial strains and growth conditions**

All *B. subtilis* strains used in this work are derived from the laboratory wild type strain 168. They are listed in Table S2. *Bacillus subtilis* was grown in LB (Lysogeny broth) medium, SP (sporulation) medium and in C minimal medium containing glucose and ammonium as basic sources of carbon and nitrogen respectively (Commichau et al., 2008). Minimal medium was supplemented with auxotrophic requirements (at 50 mg L\(^{-1}\)) and amino acids as indicated. Plates were prepared by the addition of 17 g Bacto agar/l (Difco) to the liquid medium. *Escherichia coli* DH5\(\alpha\) and JW3060 (Sambrook et al., 1989; Baba et al., 2006) were used for cloning and complementation experiments respectively. JW3060 was grown in M9 minimal medium (Sambrook et al., 1989) with glucose (1% w/v) as the carbon source, but lacking casamino acids. Serine was added as indicated. For the determination of the tolerated serine concentrations, bacteria were grown in C glucose minimal medium to an OD\(_{600}\) of 1.0 and plated on C-Glc plates containing a wide range of serine concentrations (1–100 mM). The growth was compared after incubation of the plates at 37°C for 48 h.

**DNA manipulation and genome sequencing**

Plasmid DNA extraction from *E. coli* were performed using standard procedures (Sambrook et al., 1989). Restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. Fusion DNA polymerase (Biozym, Germany) was used for the polymerase chain reaction as recommended by the manufacturer. DNA fragments were purified using the Qiaquick PCR Purification kit (Qiagen, Germany). DNA sequences were determined using the dideoxy chain termination method (Sambrook et al., 1989). All plasmid sequences were determined using the dideoxy chain termination method (Sambrook et al., 1989). All plasmid sequences were determined using the dideoxy chain termination method (Sambrook et al., 1989). All plasmid
inserts derived from PCR products were verified by DNA sequencing. Chromosomal DNA of *B. subtilis* was isolated as described (Commichau et al., 2008). To identify the mutations in the suppressor mutant strains GP2324, GP2969, GP2970, GP2971 and GP2972 (see Table S2), the genomic DNA was subjected to whole-genome sequencing (Reuß et al., 2019). Briefly, 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, New Zealand) (Kearse et al., 2012). 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.

**Transformation and phenotypic analysis**

Standard procedures were used to transform *E. coli* (Sambrook et al., 1989) and transformants were selected on LB plates containing ampicillin (100 μg ml⁻¹). *Bacillus 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⁻¹), kanamycin (Km 5 μg ml⁻¹), spectinomycin (Spc 150 μg ml⁻¹), or erythromycin plus lincomycin (Em 25 μg ml⁻¹ and Lin 25 μg ml⁻¹).

In *B. subtilis*, amylase activity was detected after growth on plates containing nutrient broth (7.5 g L⁻¹), 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 LB medium or in C glucose medium supplemented with serine as indicated. Cells were harvested at OD₆₀₀ of 0.6 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 min⁻¹ at 28°C.

**Construction of deletion mutants**

Deletion of amino acid transporter and biosynthetic genes was achieved by transformation with PCR products constructed using appropriate oligonucleotides to amplify DNA fragments flanking the target genes and intervening antibiotic resistance cassettes (Guérout-Fleury et al., 1995) as described previously (Wach, 1996).

**Whole-genome growth phenotype screen**

The screen was carried out as described previously (Koo et al., 2017) with modifications that optimized screening for serine toxicity. Plates for screening were allowed to dry for 2 days. The BKE (Erm³) library was arrayed in 384-well plates using a Biomek FX liquid handling robot (Beckman Coulter) and stored as glycerol stock. To screen the whole BKE library, cells were pinned from glycerol stocks onto rectangular LB agar plates in 384-format using a Singer Rotor robot, then four 384-format plates were combined and pinned to 1536-format. For each screen, exponentially growing cells in 1536-format were then pinned to glucose minimal agar plates (growth control) and glucose minimal plates supplemented with three different concentrations of L-serine (0.38, 0.75 and 1.5 mM). Then, plates were incubated at 37°C in a humidified incubator for about 24–44 h. Plates were imaged using a Powershot G10 camera (Canon) and serine-resistant mutants were identified by their position in the plates. Each mutant was confirmed by sequencing of their barcodes.

**Plasmids**

Plasmid pAC5 (Martin-Verstraete et al., 1992) was used to construct translational fusions of the ybeC, sdaAB and hom control regions with the lacZ gene. For this purpose, the regions upstream of these genes were amplified using appropriate oligonucleotides. The PCR products were digested with EcoRI and BamHI PCR and cloned into pAC5 linearized with the same enzymes. The resulting plasmids were pGP2287 (*ybeC*), pGP2295 (*sdaAB*), pGP2294 (*sdaAB*) and pGP2296 (*hom*).

For the expression of YbeC in *E. coli*, we constructed plasmid pGP2987. For this purpose, the *ybeC* gene was amplified using chromosomal DNA of *B. subtilis* as a template. The PCR product was digested with BamHI and Sall and cloned into the expression vector pWH844 (Schirmer et al., 1997).

For the expression of the threonine dehydratase IivA in *B. subtilis*, plasmid pGP2289 was constructed by cloning a DNA fragment covering the ilvA gene between the BamHI and Sall restriction sites of the overexpression vector pBQ200 (Martin-Verstraete et al., 1994).

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Supporting Information
Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:
Table S1 Known and potential amino acid transporters in B. subtilis
Table S2. Bacillus subtilis strains used in this study.