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Aspartate deficiency limits peptidoglycan synthesis and sensitizes cells to antibiotics targeting cell wall synthesis in *Bacillus subtilis*

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Summary

Peptidoglycan synthesis is an important target for antibiotics and relies on intermediates derived from central metabolism. As a result, alterations of metabolism may affect antibiotic sensitivity. An *aspB* mutant is auxotrophic for aspartate (Asp) and asparagine (Asn) and lyses when grown in Difco sporulation medium (DSM), but not in LB medium. Genetic and physiological studies, supported by amino acid analysis, reveal that cell lysis in DSM results from Asp limitation due to a relatively low Asp and high glutamate (Glu) concentrations, with Glu functioning as a competitive inhibitor of Asp uptake by the major Glu/Asp transporter GltT. Lysis can be specifically suppressed by supplementation with 2,6-diaminopimelate (DAP), which is imported by two different cystine uptake systems. These studies suggest that aspartate limitation depletes the peptidoglycan precursor meso-2,6-diaminopimelate (mDAP), inhibits peptidoglycan synthesis, upregulates the cell envelope stress response mediated by σ^M and eventually leads to cell lysis. Aspartate limitation sensitizes cells to antibiotics targeting late steps of PG synthesis, but not steps prior to the addition of mDAP into the pentapeptide sidechain. This work highlights the ability of perturbations of central metabolism to sensitize cells to peptidoglycan synthesis inhibitors.

Abbreviated summary

Peptidoglycan synthesis relies on intermediates derived from central metabolism. Cells experiencing aspartate deficiency are compromised in their ability to synthesize meso-diaminopimelate, a peptidoglycan precursor, and are therefore sensitized to antibiotics targeting late steps of PG synthesis.

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Introduction

Bacillus subtilis is a metabolically versatile soil bacterium and can synthesize all 20 amino acids. Amino acids are essential for synthesis of proteins and other macromolecules, and many can also be catabolized to yield intermediates of the tricarboxylic acid (TCA) cycle. Amino acid metabolism is governed by master regulators, such as CcpA, TnrA, and CodY, working in concert with pathway-specific regulation by transcriptional, translational and post-translational mechanisms in a complex, interlinked network (Geiger & Wolz, 2014, Brantl & Licht, 2010, Sonenshein, 2007).

Glutamate (Glu) serves as a central link between nitrogen and carbon metabolism and, together with aspartate, functions as a key branch point amino acid linking amino acid biosynthesis with central carbon metabolism (Figure 1). Glutamate is synthesized by glutamate synthase (also known as glutamine oxoglutarate aminotransferase, GOGAT), encoded by *gltAB* in *B. subtilis*, which converts one molecule each of Gln and α-ketoglutarate into two molecules of Glu. Glutamate can be imported into the cell by GltT (Zapras et al., 2015), GltP (Tolner et al., 1995) and YveA (Lorca et al., 2003).

Glutamate homeostasis is tightly regulated in *B. subtilis* (Gunka & Commichau, 2012). Expression of *gltAB* is under control of GltC, which senses the ratio between Glu (the product) and α-ketoglutarate (the substrate) (Picossi et al., 2007). When growing in medium containing glucose and ammonium, cells are rich in TCA cycle intermediates and GltC is bound to α-ketoglutarate and activates expression of *gltAB* (Commichau et al., 2007, Picossi et al., 2007). When intracellular Glu is high, Glu binds to GltC and represses *gltAB* (Picossi et al., 2007). In the presence of Arg or other amino acids that can be metabolized into Glu, the glutamate dehydrogenase RocG is induced and degrades Glu into α-ketoglutarate and ammonium (Belitsky & Sonenshein, 1998). GltC can also physically interact with RocG and it was suggested that this interaction affects GltC activity (Commichau et al., 2007, Picossi et al., 2007). *B. subtilis* 168 contains two genes encoding glutamate dehydrogenases, a functional *rocG* gene and cryptic *gudB* gene (Belitsky & Sonenshein, 1998). When a *rocG* null mutant grows on complex sporulation medium, there is strong selection for mutations that regenerate functional *gudB* (Gunka et al., 2012), highlighting the importance of maintaining Glu homeostasis in the cell. In addition to serving as a major nitrogen donor and

anion in the cell and a precursor for protein synthesis, Glu is also important for cell wall synthesis. D-glutamate, synthesized by glutamate racemase from L-glutamate, is incorporated into the pentapeptide side-chain of the peptidoglycan (PG) precursor lipid II. Mutations that affect Glu homeostasis have been found to affect resistance to antibiotics targeting early (fosfomycin) and late (beta-lactams) stages of PG synthesis (Lee *et al.*, 2012).

In contrast with Glu, synthesis of Asp is not known to be tightly regulated. The main pathway of Asp synthesis in *B. subtilis* is through aspartate transaminase AspB, which transfers an amino group from Glu to oxaloacetate to form Asp (Figure 1) (Ochi *et al.*, 1981). AspB is constitutively and highly expressed, and is one of the most abundant proteins in vegetative cells (Eymann *et al.*, 2004). Asp is imported by GltT, GltP and YveA (Zapras *et al.*, 2015), which are also involved in Glu uptake. In *B. subtilis*, Asp can also be generated from Asn by L-asparaginases AnsA and AnsZ (Sun & Setlow, 1991). However, their regulation indicates that these asparaginases function in degradation of Asn (and then Asp) ultimately yielding fumarate (Daniel & Errington, 1993; Fisher & Wray, 2002).

Aspartate is the starting point for synthesis of the Asp family amino acids, which include Asn, Lys, Thr, Met and Ile. The pathway that generates Lys includes both meso-diaminopimelate (mDAP), which is used in PG synthesis in many bacteria, and a branch point to dipicolinate, important for heat resistance of endospores (Daniel & Errington, 1993; Chen *et al.*, 1993) (Figure 1). The regulation of Asp usage mostly results from feedback inhibition (Dumas *et al.*, 2012). For example, three aspartokinases convert L-aspartate into L-aspartyl-4-phosphate, the first committed step for generating Asp family amino acids (except Asn). Aspartokinase I (DapG) is feedback inhibited by mDAP but not L,L-diaminopimelate (DAP) (Rosner & Paulus, 1971; Chen *et al.*, 1993), aspartokinase II (LysC) by Lys (Zhang *et al.*, 1990), and aspartokinase III (ThrD) by the simultaneous presence of Thr and Lys (Kobashi *et al.*, 2001; Graves & Switzer, 1990). The branch point enzyme homoserine dehydrogenase (Hom) initiates the synthesis of Met, Thr, and Ile, and can be feedback inhibited by these products (Gutierrez-Preciado *et al.*, 2009).

In bacteria, the amino acid composition of the stem pentapeptide of PG is typically L-Ala₁-D-Glu₂-mDAP₃-D-Ala₄-D-Ala₅, with the third amino acid being L-lysine in some bacteria (Zhao *et al.*, 2017). Whether mDAP₃ or L-Lys₃ is used is mainly dependent on the substrate specificity of the corresponding ligase (MurE), as well as the cytoplasmic concentration of L-lysine versus mDAP (Ruane *et al.*, 2013). Because of its essential role in PG synthesis, the stem pentapeptide serves as an important target for antibiotics including D-cycloserine, vancomycin and teicoplanin. D-cycloserine is a mimic of D-Ala, and inhibits D-Ala-D-Ala synthesis by inhibiting both alanine racemase (Alr) and D-Ala-D-Ala ligase (Ddl) (Strominger *et al.*, 1960). Vancomycin inhibits PG synthesis by binding to the D-Ala₄-D-Ala₅ termini of stem peptides thereby preventing transpeptidation (Arthur *et al.*, 1996).

In this work, we characterized the growth properties of a *B. subtilis* *aspB* mutant in a variety of nutrient conditions, which revealed that Asp limitation under otherwise nutrient rich conditions leads to depletion of mDAP and cell lysis. Aspartate limitation is exacerbated by high concentrations of Glu, which competitively inhibits Asp import. Depletion of mDAP can be rescued by addition of its precursor DAP, which is imported through two cystine

uptake systems, TcyABC and TcyP. Under these conditions, Asp deficiency sensitizes cells to antibiotics targeting late steps of PG synthesis, thereby highlighting the intimate connection between central metabolism, PG synthesis and antibiotic susceptibility.

Results

An aspartate transaminase mutant lyses on Difco sporulation medium (DSM)

Moenomycin (MOE) is an antibiotic that inhibits the transglycosylase activity of class A PBPs for peptidoglycan synthesis (Figure 1). During a transposon library selection for MOE resistant (MOE^R) mutants, we identified multiple independent insertions in *ypmB* (Hachmann, 2010), encoding a membrane protein of unknown function. A *ypmB* mutant has recently been shown to suppress the tetracycline sensitivity of an *ezrA* mutation (and the name *tseB* was therefore proposed) and has a reduced cell length (Gamba *et al.*, 2015). However, the physiological function of YpmB remains unknown.

Interestingly, when grown on Difco sporulation medium (DSM) plates (a medium that induces sporulation), a *ypmB::mIs* mutant exhibited pronounced cell lysis (Guariglia, 2013). Since *ypmB* is the second gene in a three-gene operon (*ypmA-ypmB(tseB)-aspB*) (Figure 2A), we tested if the MOE^R and DSM lysis phenotypes were due to a polar effect on the downstream *aspB* gene. The MOE^R phenotype of the *ypmB::mIs* mutant can be suppressed by ectopic expression of *ypmB* (cells become MOE sensitive), but not *aspB* (Figure 2B). Indeed, ectopic expression of *aspB* increased MOE^R rather than restoring sensitivity (Figure 2B). In contrast, the DSM lysis phenotype was suppressed by *aspB*, but not *ypmB* (Figure 2C, D). We conclude that MOE^R results from lack of *ypmB*, but the DSM lysis phenotype results from reduced expression of *aspB*. The mechanism underlying the increased MOE^R of the *ypmB* mutant strain is not yet clear, but we note that YpmB is predicted to have PEPSY domains that are implicated in regulation of peptidase and perhaps other enzymatic activities of cell-membrane localized proteins (Yeats *et al.*, 2004, Gamba *et al.*, 2015). Here, we focus on the role of AspB in preventing cell lysis on DSM.

Since the DSM lysis phenotype of the *ypmB::mIs* mutant strain appeared to result from polarity, we next tested the growth properties of an *aspB* null mutant. When grown on a DSM plate at 37°C overnight, an *aspB* null mutant lysed and this phenotype was complemented by ectopic expression of *aspB* (Figure 2C). When grown in liquid DSM, an *aspB* null mutant grew to OD₆₀₀ ~0.25 and lysed back to OD₆₀₀ ~0.1 (Figure 2D), suggestive of nutrient limitation. In comparison, an *aspB* mutant grew almost as well as the *B. subtilis* 168 parent strain (WT) in LB medium (Figure 2B), which is richer in amino acids (Table 1, SI Datasheet).

The *aspB* gene encodes aspartate transaminase (interconverting L-glutamate and oxaloacetate with L-aspartate and α-ketoglutarate; Figure 1), and an *aspB* null mutant is an Asp/Asn auxotroph (Figure S1A, B), as previously reported (Ochi *et al.*, 1981). Supplementation of DSM with 10 mM Asp (final concentration) greatly increased cell yield and completely suppressed lysis (Figure 3A), suggesting that limitation for Asp may trigger lysis. Asn can be converted to Asp by two asparaginases, AnsA and AnsZ (Fisher & Wray,

2002). Consistently, addition of 10 mM Asn (final concentration) also suppressed the lysis phenotype in DSM (Figure 3A).

Lysis of *aspB* results from depletion of Asp and competition with Glu for GltT

Deletion of *aspB* blocks both the synthesis of Asp and the consumption of Glu, and under conditions of limiting Asp supply from the medium could lead to either depletion of Asp or accumulation of Glu in the cells. Because of the importance of Glu homeostasis in *Bacillus subtilis*, we first investigated whether the lysis of *aspB* on DSM is due to intracellular accumulation of Glu. Indeed, addition of 10 mM Glu (final concentration) to DSM medium reduced the peak OD₆₀₀ of the *aspB* null mutant from ~0.25 to ~0.15 (Figure 3A). Furthermore, in minimal medium (MM) high Glu antagonized the ability of Asp to support *aspB* growth (Figure S1C). In *B. subtilis*, Gln is a preferred nitrogen source and can be readily converted into Glu (Detsch & Stulke, 2003). However, a high concentration of Gln (5 or 10 mM) did not have a similar inhibitory effect (Figure 3A, S1C), although under this condition intracellular Glu levels should also be elevated. This led us to hypothesize that rather than being toxic itself, due to intracellular accumulation, Glu was acting as an antagonist of Asp import.

Glu and Asp can be imported by three Glu/Asp transporters, GltP, YveA and GltT (Tolner *et al.*, 1995, Lorca *et al.*, 2003), with GltT being the major importer for both amino acids (Zaprasis *et al.*, 2015). To test whether growth inhibition of the *aspB* mutant by Glu might be caused by competitive inhibition of Asp import, we mutated each of the three Asp/Glu importers in an *aspB* null mutant to see if the deletion of an importer would mimic the addition of Glu. Although deletion of *gltP* or *yveA* in the *aspB* mutant did not cause a noticeable growth defect, inactivation of *gltT* greatly reduced the growth yield (peak OD₆₀₀) of the *aspB* mutant (Figure 3B), similar to that seen for the *aspB* single mutant growing in DSM supplemented with Glu (Figure 3A). Thus, direct inactivation of Asp uptake by deleting *gltT* mimics the presence of high Glu, which may competitively inhibit uptake of Asp by GltT.

To further demonstrate that lysis is affected by relative concentrations of Asp and Glu in the medium, we altered cells to increase consumption of Glu. Glutamate is degraded by glutamate dehydrogenases RocG and GudB (Commichau *et al.*, 2008). We reasoned that degradation of intracellular Glu may allow cells to deplete Glu from the medium and facilitate Asp uptake. Indeed, when RocG was overexpressed from an IPTG inducible P_{spac(hy)} promoter it completely suppressed the lysis phenotype of the *aspB* null mutant, and the final OD₆₀₀ increased from ~0.25 to about 0.35 (Figure 3C), possibly due to the ability of cells to more efficiently access Asp from the medium. Similarly, deletion of glutamate synthase GltA, which prevents Glu synthesis, also rescued the *aspB* lysis phenotype (Figure 3C). If these effects of altering Glu metabolism are due to effects on Asp import, then these mutations should be epistatic with *gltT*. Indeed, in a *gltT* null background, overexpression of *rocG* or deletion of *gltA* could no longer rescue *aspB* lysis (Figure 3D). This is consistent with the hypothesis that consumption of Glu facilitates Asp uptake.

This model makes several predictions about the levels of Glu and Asp in DSM medium (in which *aspB* cells lyse) and LB medium (in which they do not), and how these levels might

be affected by mutations in central metabolism. Using amino acid analysis (Table 1, SI datasheet), we found that DSM is relatively poor in Asp (0.14 mM free Asp without hydrolysis and 1.68 mM with hydrolysis, as hydrolysis releases free amino acids from oligopeptides) compared to LB (0.55 mM and 3.39 mM, respectively), consistent with published work (Sezonov *et al.*, 2007). These differences in amino acid levels are reflected in the growth yield (final OD₆₀₀) of the *aspB* mutant in the two media (Figure 2B, D), and are generally consistent with the dependence of growth yield on Asp supplementation as observed in MM (Figure S1A). In addition, DSM contains much more Glu (0.93 mM without and 2.90 mM with hydrolysis) than Asp. Since GltT has a similar affinity for these two amino acids (Zaprasis *et al.*, 2015), it is likely that Glu competitively inhibits Asp uptake under conditions of our experiments.

This model is further supported by analysis of residual free amino acids after 24 hours of growth of the WT, *aspB*, *aspB gltA* and *aspB* P_{spac(hy)}-*rocG* mutants in DSM. WT cells, which grew to high density (Figure 3C), deplete both free Asp (0.05 mM remaining) and Glu (0.1 mM remaining) (Figure 3E). In contrast, the *aspB* mutant failed to grow to high density (Figure 3C) and there was considerable Asp (0.16 mM) and high Glu (1.4 mM) in the spent medium (Figure 3E). In *aspB* mutant cells overexpressing RocG, Glu in the medium was reduced to 0.21 mM and Asp to 0.04 mM, similar to WT (Figure 3E). Deletion of *gltA* also reduced residual Glu in a similar but less dramatic manner, and correspondingly increased the ability of cells to utilize Asp (Figure 3E). The additional Asp rendered accessible by lowering Glu is not large (estimated at ~0.1 mM). This is consistent with the small increase of final OD₆₀₀ when *rocG* is overexpressed (from OD₆₀₀ 0.25 to 0.4, Figure 3C), possibly because of limited Asp in DSM. Collectively, these results suggest that high exogenous Glu competitively inhibits Asp uptake by GltT, thereby exacerbating Asp limitation of the *aspB* mutant in the relatively Asp-poor DSM.

aspB lysis is caused by depletion of mDAP

In addition to being used for protein synthesis, Asp is used for the synthesis of Lys, Thr, Met, and Ile, as well as mDAP for peptidoglycan synthesis and dipicolinic acid for endospores (Figure 1). We next tested whether supplementation with any of these metabolites could suppress lysis in DSM. Supplementation with DAP (also known as L, L-diaminopimelic acid or 2,6-DAP, a precursor of mDAP) completely suppressed lysis and allowed cells to achieve a final OD₆₀₀ of ~0.5 (Figure 4A), close to WT in this medium (maximal OD₆₀₀ of 0.7-0.8; Figure 4B).

We hypothesized that DAP supplementation was providing mDAP for PG synthesis (Figure 1), and thereby suppressing cell lysis. However, DAP is also a precursor for Lys. In contrast with DAP, cells grown in DSM supplemented with Lys still showed an initial lysis before a gradual recovery (Figure 4A). The slow recovery was not specific to Lys as we also observed some recovery when supplemented with Ile or Trp (Figure 4A). Although the WT *B. subtilis* strain 168 used in this work is a tryptophan auxotroph, Trp is unlikely to be limited for cell growth, as DSM contains ~0.06 mM Trp (Table 1), which is over ten times higher than the required amount (~0.005 mM) for WT to grow to an OD₆₀₀ of ~0.6 in minimal medium (Figure S2A). In addition, supplementing DSM with up to 10 mM Trp did not significantly

increase cell yield (Figure S2B). Thus, both Lys and Trp facilitate recovery of the *aspB* mutant in a dose dependent manner after lysis, but have no effect on lysis itself (Figure S3). We speculate that this effect may result from increased nutrition supply to the not yet lysed cells, which facilitates the cannibalization of Asp from lysed cells. Indeed, supplementation with TCA cycle intermediates also facilitates post-lysis recovery, but has no effect in preventing lysis (Figure S4). Overall, the much more dramatic restoration of growth and complete prevention of lysis with DAP compared with Lys suggest that growth limitation and lysis results from a lack of mDAP for PG synthesis.

We also noticed a modest effect in suppressing lysis by supplementation with Met (Figure 4A). Met can be synthesized from Asp through a pathway starting with the branch point enzyme homoserine dehydrogenase (Hom) (Figure 1). Hom converts L-aspartate semi-aldehyde into homoserine, the precursor for synthesis of Met, Thr, and Ile, and can be feedback inhibited by Met (Gutierrez-Preciado *et al.*, 2009). We hypothesized that the partial rescue effect from Met may result from inhibition of Hom, which would spare L-aspartate semi-aldehyde for the DapA pathway and synthesis of mDAP (Figure 1). Because DSM contains all the amino acids produced by the Hom pathway (Table 1, SI datasheet), we can delete *hom* under this growth condition (Figure 4B) and test whether this allows a redirection of L-aspartate semi-aldehyde into the DAP pathway. Indeed, deletion of *hom* completely abolished lysis in the *aspB hom* double mutant (Figure 4B). Overexpression of DapA using an IPTG-inducible promoter led to a similar phenotype (Figure 4B), likely because it diverts more L-aspartate semi-aldehyde into the DAP pathway. In contrast, overexpression of Asd, the aspartate semi-aldehyde dehydrogenase that produces the common substrate for Hom and DapA, did not have much effect (Figure 4B). SpoVFAB synthesizes dipicolinic acid, an abundant component of endospores, and is a major pathway that consumes precursors for mDAP in sporulating cells (Daniel & Errington, 1993). Since this pathway is not active in vegetative cells, deletion of *spoVFA* had no effect on the *aspB* lysis phenotype (Figure 4B).

Amino acid and mDAP limitation are physiologically distinct

An *aspB* mutant grown in DSM supplemented with 1 mM DAP temporarily ceases growth at roughly the same cell density as in the absence of DAP (OD₆₀₀ ~0.3), but lysis (as defined by the loss of optical density) was inhibited and slow growth resumed to a final OD₆₀₀ of ~0.45 after 20 hours (Figure 5A). With 10 mM DAP, cells grew to this same final OD₆₀₀ after only 8 hours (Figure 5A). The similar final cell yield suggests that growth yield is ultimately determined by availability of Asp in the medium. Since the *aspB* mutant cannot grow to the same cell density as WT, even when supplemented with DAP (Figure 5B), we infer that the lowered growth rate and cell yield is due to insufficient Asp (or Asp family amino acids) for protein synthesis. We hypothesize that the growth rate stimulation with 10 mM compared to 1 mM DAP (Figure 5A) may be due to the known ability of mDAP to inhibit aspartokinase I (DapG) (Graves & Switzer, 1990, Rosner & Paulus, 1971), which will reduce the amount of Asp used to synthesize other Asp family amino acids.

In addition to its role in Asp synthesis, AspB could in principle function as an anaplerotic enzyme by providing α -ketoglutarate to sustain TCA cycle function. To test whether this

might be a cause for the reduced cell yield, we tested the effect of supplementation with both DAP and 10 mM of various TCA cycle intermediates. None of the tested intermediates increased yield beyond that seen with DAP alone (Figure 5B), consistent with the idea that reduced growth of the *aspB* mutant under these conditions is due to an insufficiency of Asp to support protein synthesis. Consistent results were observed in MM: an *aspB* mutant grown in MM supplemented with a limiting amount of Asp (1 mM) did not display an increase in cell yield when DAP was added (Figure S1D).

When an *aspB* mutant is grown in the presence of 10 mM DAP, cells no longer lyse, and are likely growth limited by the requirement of Asp and Asp family amino acids for protein synthesis (Figure 1). This limitation can be exacerbated by removal of the major Asp uptake system, GltT. In the presence of DAP the *aspB gltT* double mutant grew slowly to OD₆₀₀ ~0.5 (Figure 5C). The similar final OD₆₀₀ for the *gltT*⁺ and *gltT* strains suggests that the remaining Asp uptake system(s), presumably GltP and YveA (Tolner *et al.*, 1995, Lorca *et al.*, 2003), can still import Asp from the medium, albeit at a lower rate. However, these Asp uptake systems are still sensitive to Glu competition, as addition of 10 mM Glu completely abolished *aspB gltT* growth, even in the presence of DAP (Figure 5C). Overall, these data strongly suggest that when DAP is provided, growth and cell yield of the *aspB* mutant is determined by the availability of Asp for protein synthesis, whereas when mDAP is limited cell lysis results from a deficiency in PG synthesis.

In contrast to the lysis observed in DSM, the *aspB* mutant did not lyse in MM when a low concentration of Asp was supplemented (0.5 mM or 1 mM, Figure S1A). One notable difference between these media is that in MM cells are synthesizing most amino acids and are only provided with Trp (0.24 mM) and a limiting amount of Asp, whereas DSM contains all 20 proteinaceous amino acids (Table 1). Indeed, when additional amino acids were supplemented into MM, either in the form of casamino acids (CAAs) or a mixture of 17 amino acids (17AAs; the 20 essential amino acids omitting Asp, Asn and Glu), the *aspB* mutant exhibited rapid lysis after reaching its peak OD₆₀₀ (Figure 5D). We hypothesized that lysis might be correlated with growth rate, with fast-growing cells having more active autolysins and therefore being more vulnerable to a disruption in PG synthesis (Mah & O'Toole, 2001, Tuomanen *et al.*, 1986). Indeed, under growth conditions that caused lysis of the *aspB* mutant (DSM, or in MM supplemented with 0.5% CAA or 17AAs) the *aspB* mutant grew at a rate similar to, or even faster than, that of WT in DSM until Asp was depleted (Figure 5E). In comparison, when the *aspB* mutant was grown in MM with limiting Asp and ammonium as nitrogen source, cells grew slower and did not lyse after Asp depletion. Overall, our results suggest a correlation between growth rate and lysis, with rapid growth contributing to lysis upon mDAP depletion in the cell.

DAP can be imported into *B. subtilis* by cystine uptake systems TcyABC and TcyP

Previous studies suggest that in *E. coli* and *Salmonella* Typhimurium, DAP can be imported by cystine uptake systems, likely due to their structural similarity (Leive & Davis, 1965, Stephen & Nicholas, 1986) (Figure 6A). There are two cystine uptake systems in *E. coli* (Berger & Heppel, 1972), with TcyJLN able to uptake DAP and TcyP more specific for cystine (Chonoles Imlay *et al.*, 2015). Uptake of DAP in *Bacillus megaterium* was resistant

to the presence of cystine, suggesting the uptake is not through cystine import pathways (Gally *et al.*, 1991). However, in the same study *Bacillus subtilis* 168 exhibited no detectable DAP uptake with or without cystine (Gally *et al.*, 1991), in contrast to our observation that DAP can suppress the lysis of the *aspB* mutant.

To investigate if DAP is imported into the cells using cystine uptake systems in *B. subtilis*, we used the lysis phenotype of the *aspB* mutant as a readout and tested whether cystine can abolish the rescue of lysis by DAP. With supplementation with 600 μ M cystine, 1 mM DAP could no longer prevent the *aspB* lysis (Figure 6B), suggesting that *B. subtilis* can indeed import DAP and this uptake is likely through cystine uptake pathways. However, the import is likely to be slow and unable to replace endogenous DAP synthesis, as supplementation with 10 mM DAP cannot bypass the essentiality of genes (such as *dapB* and *dapL*) upstream of DAP synthesis in the mDAP/lysine pathway, as tested using CRISPRi based gene knockdown strains (Peters *et al.*, 2016), either in LB and DSM (data not shown), or in minimal medium (no cystine to compete with DAP import) supplemented with 3.4 mM Lys (Figure S5).

In *B. subtilis*, there are three cystine uptake systems: TcyABC, TcyJKLMN and TcyP (Burguiere *et al.*, 2004). TcyABC and TcyJKLMN are ATP binding cassette (ABC) transporters and TcyP is a proton-cystine symporter. To determine which of these three cystine uptake systems are involved in DAP import, we tested mutant strains lacking one, two, or all three systems for the ability of DAP to prevent lysis of the *aspB* mutant (Figure 6C). The results suggest that TcyABC and TcyP are the two major DAP uptake systems, with TcyJKLMN playing little if any role. The limited role for TcyJKLMN was surprising since import of L-[¹⁴C]-cystine by this system is known to be partially inhibited by mDAP (Burguiere *et al.*, 2004). Regardless, our results suggest that TcyJKLMN does not import sufficient DAP to suppress lysis and support growth under these conditions.

Depletion of mDAP causes cell wall stress and cell morphology defects

Since mDAP is an essential substrate for PG synthesis, mDAP limitation is predicted to impair PG synthesis ultimately leading to cell lysis. Indeed, microscopic images revealed many bulged cells and cell debris when the *aspB* mutant was grown in liquid DSM, while such deformed cells were rare in the 168 WT (Figure 7A, red arrows). When supplemented with DAP, the lysis phenotype was suppressed (Figure 7B), and bulged cells were much less frequent (Figure 7E). Divalent cations such as Mg²⁺ are known to suppress PG defects, in part through altering the acetylation level of mDAP in peptidoglycan (Dajkovic *et al.*, 2017) and possibly by other mechanisms including stabilization of negatively charged cell wall through cation-anion interactions. Consistent with the idea that lysis of an *aspB* null mutant is due to lack of mDAP and weakened PG, addition of 10 mM MgSO₄ suppressed the lysis phenotype of *aspB* (Figure 7B, E).

The alternative sigma factor σ^M is known to be upregulated when PG synthesis is inhibited (Helmann, 2016). Using a P_{M-lux} luciferase reporter containing the σ^M dependent autoregulatory promoter of the *sigM* gene, we found that σ^M activity was strongly induced after 3 hours of growth in DSM medium, around the same time that growth of the *aspB* mutant ceased (Figure 7C, dotted line). However, within the first hour after growth ceased

and cells began to lyse, the σ^M activity increased another 2- to 3-fold, as measured by luciferase activity (Figure 7C, dashed line). It is worth noting that because the luciferase enzyme we used in this work has an estimated half-life of only 4.2 minutes (Radeck *et al.*, 2013) it seems that there is still ongoing protein synthesis even when the Asp pool is very limited and cells fail to generate enough mDAP for PG synthesis.

Although addition of either DAP or Mg^{2+} can suppress the lysis of the *aspB* mutant, their mechanisms are quite different: addition of DAP rescues PG synthesis by providing the limiting mDAP precursor, whereas Mg^{2+} stabilizes the cell wall without solving the underlying problem. Consistently, addition of DAP almost completely suppressed induction of σ^M while supplementation with Mg^{2+} did not (Figure 7D). We also compared these two mechanisms of suppressing lysis by monitoring cell morphology after 24 hours of growth in supplemented DSM. In WT cultures, phase-bright endospores were readily visible after 24 hours of growth in DSM, while almost no endospores were present in the *aspB* mutant. Addition of DAP enabled a smaller population to form phase-bright endospores while addition of Mg^{2+} apparently did not. Using Oufti software (Paintdakhi *et al.*, 2016), we scored more than 600 cells (exclusive of spores) for each strain and growth condition, and measured cell length and width and calculated the width to length ratio (Figure 7E, F). Insufficient peptidoglycan synthesis in the *aspB* mutant significantly changed cell shape: the *aspB* mutant was about 11% shorter and 13% wider than WT cells, causing a 27% increase in the width to length ratio (Figure 7F). Addition of DAP or Mg^{2+} did not strongly affect the width/length ratio of WT cells. However, supplementation with DAP partially reversed the shape effects in the *aspB* mutant and reduced the change in aspect ratio from a 27% to a 9% increase relative to WT. In contrast, addition of Mg^{2+} did not restore cells to a more WT shape (Figure 7F). In fact, the *aspB* mutant supplemented with Mg^{2+} displayed a further increase of the width to length ratio relative to WT (Figure 7F). These data suggest that although addition of either DAP and Mg^{2+} suppress cell lysis, the intrinsic differences between the two mechanisms result in different cell morphologies.

Limitation of mDAP sensitizes cells to cell wall antibiotics

Since the *aspB* mutant is limited for mDAP availability, we tested the effect on sensitivity against several antibiotics targeting PG synthesis. For this analysis, we used Mueller-Hinton (MH) medium in which *aspB* mutant has less of a growth defect than in DSM: single colonies grow with normal morphology on MH plates, with comparatively mild lysis apparent in the colony centers and the inoculation loading zone (Figure S6). Using MH medium, we found that an *aspB* mutant was no more sensitive than WT to fosfomycin or D-cycloserine (Figure 8A), two antibiotics targeting early steps of PG synthesis (Figure 1). However, the *aspB* mutant was significantly more sensitive to antibiotics targeting PG synthesis steps downstream of lipid II, including vancomycin and the beta-lactam antibiotic cefuroxime (Figure 8A). Addition of 10 mM Asp completely rescued the sensitivity to vancomycin and partially to cefuroxime (Figure 8B, C), consistent with the sensitivity being caused by a restricted availability of mDAP. Addition of DAP did not rescue sensitivity to vancomycin or cefuroxime (data not shown), possibly due to slow import through the cystine uptake systems. Interestingly, while addition of α -ketoglutarate together with Asp did not

have any effect on cefuroxime resistance, it reduced sensitivity to vancomycin in both WT and the *aspB* mutant background. The mechanism of this altered sensitivity is not yet clear.

Even when grown in the Asp-rich LB medium, the *aspB* mutant exhibited increased MOE^S (Figure 2B) and, consistently, our original *ypmB::mls* mutant (polar on the downstream *aspB*) can become further resistant to MOE when *aspB* is overexpressed ectopically from an IPTG inducible promoter. As a mimic for the substrate of class A PBPs (aPBPs), MOE is most potent when its aPBP targets are not bound to their true substrate lipid II. The reduced expression of *aspB* in the *ypmB::mls* mutant likely leads to partial mDAP depletion and reduced lipid II production. Experiments using a non-polar *ypmB* mutant revealed strong MOE resistance, similar to a *ypmB::mls* complemented with an ectopic copy of *aspB* (data not shown). Overall, the sensitivity of *aspB* towards vancomycin, cefuroxime and MOE demonstrates that limitation of Asp (and consequently mDAP) sensitizes cells to antibiotics targeting PG synthesis, likely due to a reduced pool of lipid II.

Discussion

It is increasingly appreciated that sensitivity to antibiotics is dependent on the metabolic state of the cell. Changes in carbon source, or from aerobic to anaerobic metabolism, can have dramatic effects on antibiotic susceptibility (Yang *et al.*, 2017, Su *et al.*, 2018). It may be possible to exploit such effects by combining antibiotics that inhibit essential cellular processes with compounds that alter metabolism to increase susceptibility. Development of new antibiotics also relies on understanding cell metabolism: for example, inhibitors of pathways presumed to be essential, such as fatty acid synthesis, may in fact be ineffective if the targeted organism does not require this pathway in the host (Brinster *et al.*, 2009, Balemans *et al.*, 2010). To complicate matters further, cell populations are heterogeneous and variations in levels of metabolites or specific proteins may determine survival after antibiotic challenge, and can partially explain the phenomenon of highly resistant persister cells (Fisher *et al.*, 2017, Shan *et al.*, 2017, Lee & Collins, 2011).

In this work, we show that an *aspB* null mutation growing in rich medium with limited Asp ceases growth and lyses due to depletion of mDAP, an essential intermediate for PG synthesis. Lysis can be prevented by chemical complementation (DAP supplementation), and this property enabled us to assign two cystine uptake systems (TcyABC and TcyP) as the major DAP importers (Figure 6, Figure S5). Lysis could also be suppressed by addition of Asp or optimized utilization of available Asp or a variety of genetic manipulations that increased the flux of limited Asp into the DAP pathway. For example, increased consumption of Glu can alleviate competitive inhibition of Asp import (largely through GltT) (Figure 3), and provision of Met can spare the branch point intermediate L-aspartate semialdehyde for entry into the DAP pathway (Figure 4). Interestingly, a recent study in *Salmonella* Typhimurium shows that a Met auxotroph (*metB*) also affects PG synthesis, and leads to a disproportionate increase relative to WT of UDP-NAM-L-Ala-D-Glu (1000-fold) compared to UDP-NAM-L-Ala-D-Glu-mDAP (5-fold) (Husna *et al.*, 2018). One possibility is that the *metB* mutation increases the amount of L-aspartate semi-aldehyde shunted into the Hom pathway for Met synthesis, thereby contributing to a depletion of mDAP and accumulation of the co-substrate for UDP-NAM-L-Ala-D-Glu-mDAP synthesis.

Because an *aspB* null mutant is unable to *de novo* synthesize Asp, cells rely on import from the medium to support protein and peptidoglycan synthesis. By measuring amino acid content in DSM and LB medium, we propose that the growth defect of the *aspB* null mutant is due to Asp limitation exacerbated by competition with Glu for Asp uptake. Our results suggest that GltT is the major Glu/Asp importer, and are in agreement with prior work (Zapras et al., 2015), even though our conditions are drastically different (our experiments are done in DSM, a complex medium containing all essential amino acids, whereas Zapras et al. used a glucose ammonium based minimal medium supplemented with 20 μ M of Glu or Asp). Growth of the *aspB* mutant can be strongly inhibited by Glu, even in the presence of DAP, suggesting that all major Asp importers are sensitive to Glu-inhibition. Interestingly, Asp uptake is crucial for host colonization of *Mycobacterium tuberculosis* (Gouzy et al., 2013a, Gouzy et al., 2013b), highlighting the importance of understanding Asp uptake systems and their role in bacterial physiology.

A major finding of this work is that the growth defect of the *aspB* mutant on DSM can be separated into two parts. In the presence of added DAP, growth limitation results primarily from amino acid limitation for protein synthesis. In contrast, when no DAP is added the initial response to Asp limitation is cell wall stress, resulting from the lack of mDAP, inducing the σ^M regulon and predisposing cells to lysis. A reduced capacity for mDAP synthesis in the *aspB* null mutant contributes to an aberrant cell morphology (Figure 7) and sensitivity to antibiotics targeting late steps in PG synthesis (Figure 8), likely caused by a limited pool of lipid II. These results are analogous to the cross-talk recently noted in *Caulobacter crescentus* in which mutations that lead to α -ketoglutarate accumulation can inhibit mDAP synthesis, leading to antibiotic sensitivity (Irnov et al., 2017).

In conclusion, in the absence of exogenous DAP, PG synthesis is the first critical process that fails as Asp is depleted (Figure 7C). The resultant cell lysis may be dependent on growth rate: when growing slowly in MM, the *aspB* mutant does not lyse after depletion of Asp and mDAP, while addition of free amino acids increases growth rate and lysis. Interestingly, a similar observation was made over 35 years ago when it was noted that the depletion of mDAP in an auxotrophic strain of *E. coli* leads to more rapid lysis in fast-growing than slow-growing cells (Leduc et al., 1982). One possible explanation is that both PG synthesizing and degrading enzymes are more active in fast growing cells than slow-growing cells, and thus the imbalance is more severe in fast-growing cells when PG synthesis is limited by mDAP depletion. Overall, this work highlights the important connection between central metabolism and cell wall synthesis and reveals how alterations in central metabolism that limit peptidoglycan sidechain biosynthesis may increase sensitivity to peptidoglycan synthesis inhibitors.

Experimental Procedures

Strains, primers, media and growth condition

All strains used in this work are listed in Table 2, and all DNA primers are listed in Table S1. Bacteria were routinely grown in liquid lysogeny broth (LB), Difco Sporulation Medium (DSM) or minimal medium (MM) with vigorous shaking, or on plates (1.5% agar; Difco) at 37 °C unless otherwise stated. LB medium contains 10 g tryptone, 5 g yeast extract, and 5 g

NaCl per liter. DSM (per 500 ml) includes 5 ml 10% KCl, 5 ml 1.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 g Bacto nutrient broth powder, 0.25 ml 1 M NaOH, and water to bring the volume to 500 ml. The medium is autoclaved at 121 °C for 20 minutes. After autoclaving, the following filter sterilized ingredients were added: 0.5 ml 1 M $\text{Ca}(\text{NO}_3)_2$, 0.05 ml 0.1 M MnCl_2 , and 10 mM FeSO_4 . The minimal medium contains 2% glucose, 2 g l^{-1} $(\text{NH}_4)_2\text{SO}_4$, 40 mM MOPS (pH 7.4), 1.2 mM K_2HPO_4 , 0.8 mM KH_2PO_4 , 1 g l^{-1} sodium citrate $\cdot 2\text{H}_2\text{O}$, 245 μM L-tryptophan, 80 nM MnCl_2 , 1 μM FeSO_4 and 0.5 mM MgSO_4 . The mixture of 17 amino acids includes alanine, arginine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. Final concentrations for each amino acid used in the growth measurement is 0.1 mg ml^{-1} . For growth curve measurements, 1 μl log phase culture ($\text{OD}_{600} \sim 0.4$) was inoculated into 200 μl of liquid medium per well in a Bioscreen 100-well plate, the plate was shaken vigorously and OD_{600} was measured every 15 minutes using an automated BioScreen growth analyzer. Plasmids were constructed using standard methods (Luo *et al.*, 2010), and amplified in *E. coli* DH5 α before transforming into *B. subtilis*. For selection of transformants, 100 $\mu\text{g ml}^{-1}$ ampicillin was used for *E. coli*. Antibiotics used for selection of *B. subtilis* transformants include: kanamycin 15 $\mu\text{g ml}^{-1}$, spectinomycin 100 $\mu\text{g ml}^{-1}$, macrolide-lincosamide-streptogramin B (MLS, contains 1 $\mu\text{g ml}^{-1}$ erythromycin and 25 $\mu\text{g ml}^{-1}$ lincomycin), and chloramphenicol 10 $\mu\text{g ml}^{-1}$.

Genetic techniques

LFH PCR, chromosomal and plasmid DNA transformation was performed as previously stated (Zhao *et al.*, 2016). The kanamycin resistance and MLS resistance cassettes were PCR amplified from plasmids pDG780 and pDG646, respectively (Guerout-Fleury *et al.*, 1995). The pPL82 plasmid-based $\text{P}_{\text{spac(hy)}}$ overexpression constructs were linearized and integrated into the *amyE* locus (Quisel *et al.*, 2001). Markerless in-frame deletion mutants (indicated by Δ in Table 2) were constructed from BKE strains as described (Koo *et al.*, 2017). Briefly, BKE strains were acquired from the Bacillus Genetics Stock Center, chromosomal DNA was extracted, and the mutation containing an *erm*^R cassette was transformed into our WT 168 strain. The *erm*^R cassette was subsequently removed by introduction of the Cre recombinase carried on plasmid pDR244, which was later cured by growing at the non-permissive temperature of 42 °C. Gene deletions were confirmed by PCR screening using flanking primers. Unless otherwise described, all PCR products were generated using *B. subtilis* 168 strain chromosomal DNA as template. DNA fragments used for gene overexpression were sequence verified. Null mutant constructions were verified by PCR. The mutant missing the entire *tcyJKLMN* operon was constructed in three steps. Briefly, a fragment containing the region upstream of *tcyJ* and adjacent *erm* cassette was amplified from BKE29380 (*tcyJ::erm*), and another fragment containing the *erm* cassette and downstream region of *tcyN* was amplified from BKE29340 (*tcyN::erm*). These two fragments were joined by overlap PCR using the shared *erm* cassette and yielded a fragment containing the upstream of *tcyJ* and the downstream of *tcyN*, with the entire *tcyJKLMN* operon replaced by an *erm* cassette. The fragment was used to transform WT strain and the *erm* cassette was later removed by the Cre recombinase encoded on plasmid pDR244. Unless otherwise stated, the *aspB* strain was used as a representative null mutant, and the *aspB::kan* or *aspB::erm* allele was used for construction of strains with multiple mutations

(Table 2). The *aspB*, *aspB::kan* and *aspB::erm* strains were compared in most growth experiments and were phenotypically indistinguishable.

Disk Diffusion Assay

Disk diffusion assays was performed as previously described (Kingston *et al.*, 2014). Briefly, overnight cultures in LB medium were inoculated into fresh LB medium and grown to exponential phase with OD₆₀₀ of 0.4. A 100 µl aliquot of each culture was mixed with 4 ml of 0.75% MH soft agar (kept at 50°C) and directly poured onto a prewarmed 37°C MH plate (containing 15 ml of 1.5% MH agar). After the soft agar solidified, a filter paper disk with diameter of 6.5 mm was placed on top of the soft agar, and an antibiotic to be tested was added to the paper disk. The plate incubated at 37°C for 20 hours. The overall diameter of the inhibition zone was measured along two pairs of orthogonal lines, and zones of inhibition are reported as the average diameter of the four measurements for each biological replicate. At least four biological replicates were used for each antibiotic and strain combination. The quantity of antibiotics used per disk is cefuroxime 10 µg, vancomycin 50 µg, D-cycloserine 500 µg, or fosfomycin 500 µg.

Amino Acid Analysis

Amino acid analysis (AAA) was performed by Metabolomics Center, Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign using GC/MS based method. For AAA of spent medium, cells were grown for 24 hours, and then removed by centrifugation at 5000 g for 10 minutes, followed by filtration of the supernatant through a 0.22 µm filter. Concentrations of free amino acids were measured using a quantitative GC/MS-based method. To measure total amino acids in media which contain oligo- and polypeptides, the medium was hydrolyzed (Davidson, 2003). Briefly, HCl was added to 6 M and samples kept at 110 °C in a sand bath for 24 hours. Hydrolyzed medium was then centrifuged at 5000 g for 10 minutes, followed by filtration through a 0.22 µm filter to remove particles formed during the hydrolysis. Hydrolyzed medium containing 6 M HCl was neutralized by NaOH to pH 7.0 before AAA.

Luciferase reporter construction and measurement

The P_M-*lux* luciferase reporter was constructed by inserting the σ^M -controlled promoter of *sigM* (P_M) amplified using primers 6808 and 6809 into the multicloning sites of pBS3C*lux* (Radeck *et al.*, 2013). The insert was confirmed by sequencing, the plasmid was linearized and integrated into the *B. subtilis* *sacA* locus. For luciferase measurements, 1 µl of exponentially growing cells were inoculated into 99 µl of fresh medium in a 96 well plate, incubated at 37 °C with shaking using a SpectraMax i3x plate reader. Because the luciferase has a short half-life (~4 minutes), OD₆₀₀ and luminescence were measured every 12 min. The data was analyzed using SoftMax Pro 7.0 software. P_M promoter activity was normalized by dividing the relative light units (RLU) by OD₆₀₀.

Phase contrast microscopy

Cells were grown in liquid medium for the time indicated and loaded on saline (0.90% NaCl, w/v) agarose pads (0.8% final concentration) on a glass slide. Phase contrast images

were taken using a Leica DMI8 microscope equipped with a 100x immersion objective and Leica Application Suite X software. Cell length and width were measured using Outfit per the software's instruction (Paintdakhi *et al.*, 2016).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Metabolic pathways relevant to this study. Cofactors such as NAD^+/NADH are not shown in the reactions for simplicity. Solid lines indicate steps requiring a single enzyme, and dashed lines indicate multiple enzymatic reactions between two compounds. Other processes, such as uptake of cystine or DAP or incorporation of dipicolinic acid into endospore, are indicated with dotted lines. Fosfomycin inhibits the synthesis of UDP-N-acetylmuramic acid (UDP-NAM) from UDP-N-acetylglucosamine (UDP-NAG) by inactivating MurAA. D-cycloserine mimics D-Ala and inhibits both the alanine racemase (Alr) and D-Ala-D-Ala ligase (Ddl), which form D-Ala-D-Ala to be added to complete the pentapeptide side chain. After synthesis at the cytoplasmic membrane, lipid II needs to be flipped to the outside of the membrane by lipid II flippase (the flipping step) and oligomerized into glycan strands (transglycosylation). The glycan strands are then crosslinked by the transpeptidase activity of penicillin binding proteins (PBP) into the mesh-like structure of peptidoglycan (transpeptidation). Vancomycin binds to the D-Ala-D-Ala motif of lipid II and prevent transpeptidation. β -lactam antibiotics such as cefuroxime (CEF) bind to PBPs and prevent transpeptidation. Mg^{2+} can bind to and stabilize the cell envelope even under conditions of reduced peptidoglycan synthesis.

Figure 2. Dissection of phenotypes caused by *ypmB* and *aspB* mutations.

A. Genetic arrangement of the *ypmAypmBaspB* operon. **B.** Representative growth curves of strains in liquid LB with or without 1 $\mu\text{g ml}^{-1}$ moenomycin. **C.** Colony morphology of strains on DSM plates after 20 hours of incubation at 37°C. **D.** Representative growth curves of strains in liquid DSM. 1 mM final concentration of IPTG are used to induce P_{spac(hy)} promoter. All growth curves were measured at least 3 times with similar results. A representative measurement is shown. Strains used were *ypmB::mIs* (HB12259), *aspB* (HB17401), *ypmB::mIs* P_{spac(hy)}-*ypmB* (HB22913), *ypmB::mIs* P_{spac(hy)}-*aspB* (HB17062), and *aspB::kan* P_{spac(hy)}-*aspB* (HB17060). Note that indistinguishable phenotypes were observed for the *aspB* and *aspB::kan* strains.

Figure 3. Lysis of the *aspB* mutant results from depletion of Asp and competition with Glu for GltT. Representative growth curves of *aspB* mutants in DSM are shown.

A. Effect of supplementation with 10 mM (final concentration) of the following amino acids: Asn, asparagine; Asp, aspartate; Glu, glutamate; Gln, glutamine. **B.** Effects of mutation of each of the known glutamate/aspartate importers. **C.** Effects of mutations affecting intracellular Glu synthesis and degradation. **D.** Effects of mutations in panel C combined with or without *gltT*. **E.** Residual concentration of Asp and Glu in DSM medium after growth of 24 hours of indicated bacterial strains, as well as acid hydrolyzed medium. The data is represented as the mean plus and minus one standard error of the mean.

Figure 4. ysis of the *aspB* mutant can be suppressed by biochemical or genetic manipulations that favor production of mDAP.

A. Representative growth curves of the *aspB* mutant in DSM supplemented with or without the following amino acids (final concentration of 10 mM each, except Trp is at 5 mM): DAP, L, L-diaminopimelate; Met, methionine; Lys, lysine; Ile, isoleucine; Thr, threonine; Trp, tryptophan. **B.** Representative growth curves of mutants in liquid DSM. 1 mM final concentration of IPTG is added to induce $P_{\text{spac(hy)}}$ promoter when present.

Figure 5. Amino acid and mDAP limitation are physiologically distinct.

A. Growth curve of the *aspB* mutant growing in DSM supplemented with 1 or 10 mM final concentration of DAP. **B.** Growth curve of WT and the *aspB* mutant in DSM supplemented with 10 mM final concentration of DAP, and with or without TCA cycle intermediates (10 mM final concentration). **C.** Growth curve of mutants in DSM supplemented with 10 mM final concentration of DAP, or 10 mM each of DAP and Glu. **D.** Growth curve of the *aspB* mutant in DSM, or MM supplemented with 0.5 mM Asp, 0.5% (w/v) casamino acids, or 0.5 mM Asp and a mixture of 17 amino acids each at concentration of 0.1 mg ml⁻¹. **E.** Growth rate of *aspB* mutant in different media. The growth rate is calculated as $\log_2(\text{OD}_{600-T_2}/\text{OD}_{600-T_1})/(T_2-T_1)$, in which T_1 and T_2 are two time points in the exponential phase of cell growth. When $\text{OD}_{600} < 0.3$, one doubling is approximated as a two-fold increase in OD_{600} value. The data is represented as the mean plus or minus one standard error of the mean, and statistically significant different samples (Student's *t* test, two-tailed $P < 0.05$) are labelled with different letters. All growth curves were measured at least 3 times and yielded similar results. A representative measurement is shown.

Figure 6. DAP can be imported through cystine uptake systems TcyABC and TcyP.
A. Chemical structure of 2,6-DAP and cystine. **B.** Growth curve of the *aspB* mutant growing in DSM with or without supplementation with DAP (1 mM final concentration), Cys (600 μM cystine), and DAP + Cys (1 mM DAP and 600 μM cystine). **C.** Growth curves of different strains in DSM supplemented with 10 mM DAP. All growth curves were measured at least 3 times and yielded similar results. A representative measurement is shown.

Figure 7. Limitation of mDAP causes cell wall stress and cell morphology defects.

A. Representative phase contrast microscopic images of WT and the *aspB* mutant after 12 hours of growth in DSM. Red arrows point to deformed or lysed cells. **B.** Growth curve of the *aspB* mutant in DSM with or without supplementation with 10 mM MgSO_4 or 10 mM DAP. **C.** σ^M activity (relative light unit (RLU)/ OD_{600} , left axis, closed symbols) and OD_{600} (right axis, open symbols) of WT and the *aspB* mutant in DSM. The dotted line indicates the time when the *aspB* mutant starts to lyse, and the dashed line indicates when the luciferase synthesis begins to decrease. Because the luciferase has a short half-life (~4 minutes), OD_{600} and luminescence were measured every 12 minutes to generate near real time gene expression data. **D.** σ^M activity (relative light unit (RLU)/ OD_{600}) of the *aspB* mutant in DSM with or without supplement of 10 mM DAP or 10 mM MgSO_4 . All growth curves were measured at least 3 times and yielded similar results. A representative measurement is shown. **E.** Representative phase contrast microscopic images of WT and the *aspB* mutant after 24 hours of growth in DSM, with or without supplementing 10 mM DAP or MgSO_4 . Red arrows point to deformed or lysed cells. **F.** Comparison of cell length, width and aspect between WT and the *aspB* mutant under different growth conditions. n is the number of cells measured per strain per condition. Percentage change of the mean compared to WT is shown above each data plot. The bottom and top of the box are the first and third quartiles, the band inside the box is the second quartile (the median), and the X inside the box is the mean. Whiskers are one standard deviation above or below the mean. Outliers are shown as single dots.

Figure 8. Limitation of mDAP sensitizes cells to antibiotics targeting late steps in PG synthesis.

A. Sensitivity of WT and the *aspB* mutant against cefuroxime, vancomycin, D-cycloserine and fosfomycin. Pairwise comparison was made to test if difference between samples are statistically significant (Student's t test, two-tailed. ***, $P < 0.01$; NS, not significant.)

B. Sensitivity of WT and the *aspB* mutant against cefuroxime when grown in MH, MH +Asp, or MH+Asp+αKG (α-ketoglutarate). **C.** Sensitivity of WT and the *aspB* mutant against vancomycin when grown in MH, MH+Asp, or MH+Asp+αKG. Pairwise comparison was made (Student's t test, two-tailed $P < 0.05$), and samples with statistically significant differences are labelled with different letters. The data is represented as the mean plus or minus one standard error of the mean, with sample number n greater-than or equal to 3.

Table 1.

Amino Acid Composition of LB and DSM

Amino Acids ^e (mM)	LB ^a	Hydrolyzed LB ^b	DSM ^a	Hydrolyzed DSM ^b
Alanine	1.47	1.78	0.38	2.72
Arginine	1.34	0.81	0.52	1.45
Asparagine	0.49	0.11 ^c	0.08	0.14 ^c
Aspartate	0.55	3.39	0.14	1.68
Glutamine	4.50	5.51 ^c	0.47	1.34 ^c
Glutamate	2.32	7.17	0.93	2.90
Glycine	0.71	1.18	0.26	5.77
Histidine	0.33	0.88	0.07	0.65
Isoleucine	0.83	3.91	0.27	0.89
Leucine	1.75	2.60	0.32	1.23
Lysine	3.53	4.74	0.42	1.16
Methionine ^d	0.40	0.82	0.01	0.01
Phenylalanine	0.99	2.96	0.37	0.75
Proline	0.33	4.54	0.13	3.12
Serine ^d	0.88	3.36	0.30	1.50
Threonine ^d	2.38	5.55	0.27	1.14
Tryptophan ^d	0.45	0.07	0.06	0.07
Tyrosine ^d	0.31	0.94	0.21	0.08
Valine	1.13	2.14	0.17	1.05

^aThe amino acid composition is measured in unhydrolyzed media. This method measures free amino acids in the medium, which includes amino acids sensitive to acid hydrolysis, but does not measure amino acids that in oligo- and polypeptides. One measurement was performed for each medium. The measurement was performed by Metabolomics Center, Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign using a GC/MS-based method.

^bThe acid hydrolysis was performed by addition of concentrated HCl into media to a final concentration of 6 M in sealed glass tubes. The mixture was placed in a sand bath at 110 °C for 24 hours for hydrolysis. The mixture was then neutralized to pH 7 by addition of NaOH. Two samples were measured for each medium and the average is shown. The measurement was performed by Metabolomics Center, Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign using GC/MS based method.

^cAsparagine and glutamine can be hydrolyzed to aspartate and glutamate, respectively. The presence of the amine form is likely due to incomplete hydrolysis.

^dMethionine, serine, threonine, tryptophan and tyrosine are known to be unstable under acid hydrolysis and the data acquired may be lower than the true value to different degrees according to the stability of each amino acid.

^eCystine is not reported due to incomplete data acquired from the measurement.

Table 2.

Strains used in this study

Number	Genotype	Reference/Construction ^a
168	Wild type <i>B. subtilis</i> strain (<i>trpC2</i>)	Lab stock
HB12259	<i>ypmB::mls</i>	LFH PCR → 168 (Guariglia, 2013) ^b
HB17053	<i>aspB::kan</i>	LFH PCR → 168 ^b
HB17058	<i>amyE::P_{spac(hy)}-aspB</i>	pPL82- <i>aspB</i> → 168
HB17060	<i>aspB::kan, amyE::P_{spac(hy)}-aspB</i>	HB17053 → HB17058
HB17062	<i>ypmB::mls, amyE::P_{spac(hy)}-aspB</i>	HB12259 → HB17058
HB17325	<i>sacA::P_M-lux</i>	pBS3 <i>Clux</i> -P _M → 168
HB17401	<i>aspB</i>	BKE22370 → 168, then the <i>erm</i> cassette removed using pDR244
HB20720	<i>amyE::P_{spac(hy)}-rocG</i>	pPL82- <i>rocG</i> → 168
HB20749	<i>amyE::P_{spac(hy)}-rocG aspB-erm</i>	BKE22370 → HB20720
HB20873	<i>gltA</i>	BKE18450 → 168, then the <i>erm</i> cassette removed using pDR244
HB22836	<i>yveA::erm</i>	BKE34470 → 168
HB20874	<i>gltA aspB::erm</i>	BKE22370 → HB20873
HB22838	<i>gltP::erm</i>	BKE02340 → 168
HB22841	<i>gltT::erm aspB::kan</i>	HB17053 → HB22851
HB22842	<i>gltP::erm aspB::kan</i>	HB17053 → HB22838
HB22843	<i>yveA::erm aspB::kan</i>	HB17053 → HB22836
HB22851	<i>gltT::erm</i>	BKE10220 → 168
HB22869	<i>tcyC</i>	BKE03590 → 168, then the <i>erm</i> cassette removed using pDR244
HB22870	<i>tcyP</i>	BKE09130 → 168, then the <i>erm</i> cassette removed using pDR244
HB22871	<i>tcyJKLMN</i>	See Experiment Procedure
HB22878	<i>tcyC tcyP</i>	BKE03590 → HB22870, then the <i>erm</i> cassette removed using pDR244
HB22879	<i>tcyC tcyJKLMN</i>	BKE03590 → HB22871, then the <i>erm</i> cassette removed using pDR244
HB22880	<i>tcyP tcyJKLMN</i>	BKE09130 → HB22871, then the <i>erm</i> cassette removed using pDR244
HB22881	<i>tcyC tcyP tcyJKLMN</i>	BKE03590 → HB22880, then the <i>erm</i> cassette removed using pDR244
HB22885	<i>spoVFA</i>	BKE16730 → 168, then the <i>erm</i> cassette removed using pDR244
HB22887	<i>hom</i>	BKE32260 → 168, then the <i>erm</i> cassette removed using pDR244
HB22890	<i>spoVFA aspB::kan</i>	HB17053 → HB22885
HB22892	<i>tcyC aspB::kan</i>	HB17053 → HB22869
HB22893	<i>tcyP aspB::kan</i>	HB17053 → HB22870
HB22894	<i>tcyJKLMN aspB::kan</i>	HB17053 → HB22871
HB22895	<i>tcyC tcyP aspB::kan</i>	HB17053 → HB22878
HB22896	<i>tcyC tcyJKLMN aspB::kan</i>	HB17053 → HB22879
HB22897	<i>tcyP tcyJKLMN aspB::kan</i>	HB17053 → HB22880
HB22898	<i>tcyC tcyP tcyJKLMN aspB::kan</i>	HB17053 → HB22881
HB22900	<i>hom aspB::kan</i>	HB17053 → HB22887
HB22910	<i>amyE::P_{spac(hy)}-ypmB</i>	pPL82- <i>ypmB</i> → 168
HB22913	<i>ypmB::mls amyE::P_{spac(hy)}-ypmB</i>	HB12259 → HB22910

Number	Genotype	Reference/Construction ^a
HB22917	<i>amyE::P_{spac(hy)}-asd</i>	pPL82- <i>asd</i> → 168
HB22919	<i>amyE::P_{spac(hy)}-dapA</i>	pPL82- <i>dapA</i> → 168
HB22921	<i>amyE::P_{spac(hy)}-asd aspB::kan</i>	HB17053 → HB22917
HB22923	<i>amyE::P_{spac(hy)}-dapA aspB::kan</i>	HB17053 → HB22919
HB22932	<i>gltA amyE::P_{spac(hy)}-rocG</i>	HB20720 → HB20873
HB22933	<i>gltA gltT-erm</i>	BKE10220 → HB20873
HB22934	<i>amyE::P_{spac(hy)}-rocG gltT::erm</i>	HB20720 → HB22851
HB22935	<i>gltA amyE::P_{spac(hy)}-rocG aspB::kan</i>	HB17053 → HB22932
HB22937	<i>gltA gltT::erm aspB::kan</i>	HB17053 → HB22933
HB22939	<i>amyE::P_{spac(hy)}-rocG gltT::erm aspB::kan</i>	HB17053 → HB22934
HB22951	<i>sacA::P_M-lux aspB::kan</i>	HB17053 → HB17325

^a“→” indicates transformation using DNA from the former (donor) into the latter (recipient).

^bMost of the open reading frame is replaced by an antibiotic cassette.