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L-2,3-diaminopropionate generates diverse metabolic stresses in *Salmonella enterica*

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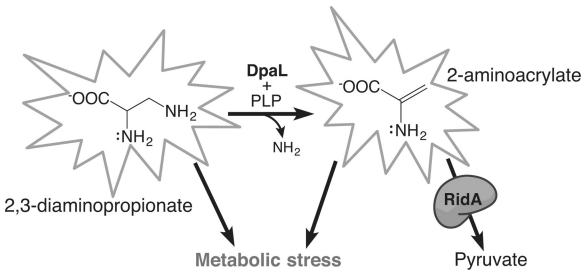
Summary

Unchecked amino acid accumulation in living cells has the potential to cause stress by disrupting normal metabolic processes. Thus, many organisms have evolved degradation strategies that prevent endogenous accumulation of amino acids. L-2,3-diaminopropionate (Dap) is a non-protein amino acid produced in nature where it serves as a precursor to siderophores, neurotoxins, and antibiotics. Dap accumulation in *S. enterica* was previously shown to inhibit growth by unknown mechanisms. The production of diaminopropionate ammonia-lyase (DpaL) alleviated Dap toxicity in *S. enterica* by catalyzing the degradation of Dap to pyruvate and ammonia. Here, we demonstrate that Dap accumulation in *S. enterica* elicits a proline requirement for growth and specifically inhibits coenzyme A and isoleucine biosynthesis. Additionally, we establish that the DpaL-dependent degradation of Dap to pyruvate proceeds through an unbound 2-aminoacrylate (2AA) intermediate, thus contributing to 2AA stress inside the cell. The reactive intermediate deaminase, RidA, is shown to prevent 2AA damage caused by DpaL-dependent Dap degradation by enhancing the rate of 2AA hydrolysis. The results presented herein inform our understanding of the effects Dap has on metabolism in *S. enterica*, and likely other organisms, and highlight the critical role played by RidA in preventing 2AA stress stemming from Dap detoxification.

Abstract



Keywords

2-aminoacrylate; RidA; enamine stress; metabolic stress

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Introduction

The robustness of a given metabolic network is influenced by the availability and reactivity of metabolites. Reactive metabolites, along with enzyme catalysts, facilitate the chemistry necessary to sustain life. However, reactive metabolites can cause damage to cellular components if they accumulate aberrantly. Systems for pre-empting damage caused by reactive metabolites often include degradation, chemical modification, compartmentalization, or excretion of the relevant stressor (Linster *et al.*, 2013; de Lorenzo *et al.*, 2014). The significance of this problem has been recognized by a recent suggestion that many genes of unknown function are involved in protecting the cell from metabolic errors (Linster *et al.*, 2013). Aberrant amino acid accumulation represents a broadly encountered form of metabolic stress. For example, human diseases such as phenylketonuria, branched-chain ketoaciduria, homocysteinuria, cystinosis, and others are associated with amino acid build-up (Wellner and Meister, 1981). In bacteria, several amino acids, including serine and cysteine, cause metabolic stress and disrupt normal metabolic processes if they accumulate (Zhang and Newman, 2008; Park and Imlay, 2003; Oguri *et al.*, 2012). Organisms such as *Escherichia coli* and *Salmonella enterica* possess independent degradative enzymes for serine and cysteine that keep the levels of these amino acids low enough to minimize stress (Zhang and Newman, 2008; Oguri *et al.*, 2012).

L-2,3-diaminopropionate (Dap) is an amino acid produced by some plants and bacteria where it serves as a precursor to secondary metabolites, including neurotoxins, antibiotics, siderophores and polyamino acids (Rao, 1978; Zhao *et al.*, 2008; Beasley *et al.*, 2011; Xu *et al.*, 2015). Chemically synthesized Dap derivatives are reported to act as potent scavengers of reactive aldehyde species, including those implicated in neurodegenerative diseases (Audic *et al.*, 2013). Recent reports describing Dap synthesis in *Staphylococcus aureus* and *Streptomyces albulus* revealed two distinct mechanisms of Dap production in bacteria, and demonstrated the importance of this metabolite in iron acquisition and poly(L-diaminopropionic acid) production, respectively (Kobylarz *et al.*, 2014; Xu *et al.*, 2015). Dap is not produced by *E. coli* or *S. enterica*, but it directly impairs growth of these organisms by unknown mechanisms (Kalyani *et al.*, 2012). The pyridoxal 5'-phosphate (PLP)-dependent degradation of Dap to pyruvate catalyzed by diaminopropionate ammonia-lyase (DpaL; EC 4.3.1.15) helped alleviate Dap stress in *E. coli* and *S. enterica* (Kalyani *et al.*, 2012). Expression of *dpaL* and protein production were specifically induced in *S. enterica* when exposed to Dap (Kalyani *et al.*, 2012). Furthermore, the narrow substrate range observed for DpaL *in vitro* and its favorable affinity for Dap (K_m = 0.1-0.3 mM) support a specific role for DpaL in degrading Dap to avoid metabolic stress (Nagasawa *et al.*, 1987; Khan *et al.*, 2003; Kalyani *et al.*, 2013).

The mechanism proposed for DpaL was reminiscent of other PLP-dependent eliminases, including serine/threonine dehydratases (IlvA/TdcB; EC 4.3.1.19) and cysteine desulfhydrase (CdsH; EC 4.4.1.1), and suggested 2-aminoacrylate (2AA) was generated as an intermediate in the degradation of Dap to pyruvate (Rajaram *et al.*, 2003; Bisht *et al.*, 2012). Strains of *S. enterica* lacking the enamine/imine deaminase, RidA (EC 3.5.99.10), were more sensitive than wild type strains to the accumulation of 2AA produced by IlvA and CdsH from serine and cysteine, respectively (Schmitz and Downs, 2004; Christopherson

et al., 2008; Flynn *et al.*, 2013; Flynn and Downs, 2013; Ernst *et al.*, 2014). Extrapolating these results suggested that RidA could be an important component of DpaL-mediated Dap detoxification in *S. enterica*.

This study was initiated to further characterize the mechanism of Dap sensitivity in *S. enterica* and to expand our current understanding of the role RidA plays in preventing 2AA stress arising from amino acid degradation. Here we show that Dap provokes a requirement for proline and directly inhibits the biosynthesis of coenzyme A and isoleucine. Furthermore, we show that the degradation of Dap by DpaL yields 2AA and exacerbates metabolic stress in the absence of RidA. The work presented here adds to mounting evidence that RidA participates broadly in amino acid detoxification pathways by preventing associated 2AA stress.

Results and discussion

L-2,3-diaminopropionate elicits a proline requirement in wild type S. enterica

Wild type *S. enterica* (DM14828) failed to grow in glucose minimal medium containing 0.25 mM Dap within 12 hours (Fig. 1a). Growth of the wild type strain resumed after 12 hours, whereas a *dpaL* mutant (DM14881) failed to grow within 24 hours, suggesting DpaL was required to overcome Dap inhibition (data not shown). To understand the growth defect, the wild type strain was embedded in soft-agar overlays on solid minimal glucose medium containing 1 mM Dap. Each of the 20 common amino acids and various vitamins were individually spotted on the agar surface. Growth stimulation was assessed after overnight incubation at 37°C. Of the supplements spotted, only L-proline stimulated growth. Liquid growth studies confirmed that 1 mM L-proline eliminated the growth inhibition caused by Dap (Fig. 1a). In addition to serving as a building block of proteins, proline behaves as an osmoprotectant in many prokaryotes and eukaryotes (Csonka, 1989). The alternative osmolyte, glycine betaine (1 mM), was added to cultures of wild type *S. enterica* grown in the presence of 0.25 mM Dap. Glycine betaine failed to restore growth in the presence of Dap (data not shown), suggesting proline acted by a mechanism other than osmoprotection.

In *E. coli* and *S. enterica*, proline biosynthesis is initiated by γ-glutamyl kinase (ProB; EC 2.7.2.11), catalyzing the phosphorylation of glutamate to generate γ-glutamyl phosphate (Fig. 1b). γ-glutamyl phosphate is labile and susceptible to nucleophilic attack, including spontaneous cyclization in solution (Gamper and Moses, 1974). ProB is thought to form an enzyme aggregate with the second enzyme in the proline biosynthetic pathway, glutamate 5-semialdehyde dehydrogenase (ProA; EC 1.2.1.41), to avoid exposure of the labile intermediate to the cellular milieu (Gamper and Moses, 1974). A previous report described variants of ProB or ProA in *E. coli* that exacerbated γ-glutamyl phosphate accumulation, leading to selective reactivity with thiol containing amino acids *in vivo* and *in vitro* (Veeravalli *et al.*, 2011). It was plausible that the primary amine attached to the β-carbon of Dap reacted with γ-glutamyl phosphate, or the subsequent proline intermediate, glutamate 5-semialdehyde. This scenario was supported by literature describing reactivity between Dap and electrophilic carbonyl groups (Audic *et al.*, 2013). The *E. coli* ProA and ProB enzymes were purified from strains harboring the appropriate ASKA (A Complete Set of *E. coli* K-12 ORF Archive) library constructs described by Kitagawa *et al.* (Kitagawa *et al.*,

2005). The impact of Dap on ProAB activity was tested *in vitro* by monitoring the conversion of glutamate (12 mM) to glutamate 5-semialdehyde based on the ProAB-dependent oxidation of NADPH (λ_{max} = 340 nm). ProAB activity was not significantly different with (6.2 ± 0.2 nmol NADPH oxidized/μM ProB min^{-1}) or without (5.6 ± 0.2 nmol NADPH oxidized/μM ProB min^{-1}) Dap (10 mM) included in the assay, indicating that Dap did not inhibit γ-glutamyl kinase or glutamate 5-semialdehyde dehydrogenase activities directly. Similar assays consisting of ProAB and glutamate (with and without NADPH) were incubated overnight in the presence of Dap (10 mM) and analyzed by HPLC or submitted for LC-MS analysis to determine if a Dap-conjugated metabolite was formed with γ-glutamyl phosphate or glutamate 5-semialdehyde. Prior to HPLC analysis, reactions were derivatized with *o*-phthalaldehyde (OPA), and peaks corresponding to glutamate (Ex: 340 nm; Em: 455 nm) and Dap (λ_{max} = 420 nm) were readily detected, while no additional peaks that would reflect a Dap-derivatized proline intermediate were detected (data not shown). Mass spectrometry similarly failed to identify a novel Dap derivative.

It was possible that Dap inhibited the final enzyme in the proline pathway, pyrroline 5-carboxylate reductase (ProC; EC 1.5.1.2). ProC catalyzes the NAD(P)H-dependent reduction of 1-pyrroline-5-carboxylate (P5C) to proline (Fig. 1b). Previous reports demonstrated that strains lacking ProC activity excreted P5C, capable of cross-feeding a *proAB* null mutant strain (Berg and Rossi, 1974). Wild type strains of *E. coli* and *S. enterica* do not excrete P5C or proline due to tight regulation of flux through the proline pathway (Berg and Rossi, 1974). A wild type *E. coli* strain carrying a feedback-resistant variant of ProB ($ProB^{D107N}$) did cross-feed a *proC* null mutant strain (Dandekar and Uratsu, 1988). Based on these phenotypic reports, experiments were designed to test whether Dap altered the cross-feeding by P5C or proline. A plasmid encoding *S. enterica* $ProAB^{D107N}$ downstream of the arabinose-inducible promoter of pBAD24 was generated. The resulting construct (pDM1462) was transformed into wild type and *proC* mutant strains, generating producer strains intended to excrete proline (DM15352) and P5C (DM15356), respectively.

A series of feeding experiments were performed and the data, some of it shown in Table 2, supported the hypothesis that Dap inhibits ProC *in vivo*. The wild type strain containing pDM1462 (DM15352) was able to cross-feed *proAB* (DM5846) and *proC* (TT9667) null mutants overlaid on glucose minimal medium (with or without DAP) containing arabinose. These data indicated that DM15352 excreted proline. The corresponding empty vector control (DM15353) failed to stimulate growth (data not shown). The *proC* mutant containing pDM1462 (DM15356) failed to cross-feed the *proC* null strain on either medium, consistent with the absence of a functional ProC (Table 2). Critically, when inoculated into a lawn of a *proAB* mutant on minimal medium, strain DM15356 both grew at the inoculation site and stimulated growth of the lawn. The growth of DM15356 indicated that a *proAB* mutant (DM5846) generated enough proline from the P5C provided (by DM15356) to feed the inoculating strain. In contrast, when Dap was added to the medium, DM15356 failed to grow at the site of inoculation or stimulate growth of the *proAB* reporter strain. The simplest explanation for these results, in conjunction with the *in vitro* experiments, is that Dap inhibited ProC in DM5846, and prevented it from a) growing, and b) producing proline to feed DM15356. The inhibition of ProC-dependent growth by Dap was always overcome by feeding proline directly. Because these observations were made *in vivo*, it is formally

possible that the inhibition of ProC by Dap occurs indirectly following an interaction between Dap and another component of the cellular milieu.

Mutants lacking DpaL are sensitive to Dap due to altered pantothenate synthetase activity

The data above indicated that proline biosynthesis is the first critical target of Dap *in vivo*. If DpaL was removed from the metabolic network, additional Dap would accumulate and potentially uncover distinct targets of Dap toxicity. Deleting the gene encoding DpaL prevented growth of *S. enterica* in the presence of 0.25 mM Dap and 1 mM proline (Fig. 2). Nutrient requirements of the *dpaL* mutant strain (DM14881) were determined on solid minimal glucose medium containing 1mM Dap and 1 mM proline. Only the coenzyme A precursor, pantothenate, stimulated growth. Liquid analysis corroborated this phenotype and showed that pantothenate (100 μM), in addition to proline, was required for growth of a *dpaL* mutant strain in the presence of Dap (Fig. 2). These data were consistent with the absence of DpaL causing increased accumulation of Dap that led to the inhibition of pantothenate biosynthesis, in addition to proline biosynthesis.

In bacteria, pantothenate is generated by pantothenate synthetase (PanC; EC 6.3.2.1) through the ATP-dependent ligation of β-alanine and pantoate. PanC was considered a potential target of inhibition by Dap due to the similarity of β-alanine and Dap, which differ by a single amino group bound to the α-carbon of Dap. An untargeted screen of amino acid inhibitors of PanC from *E. coli* offered preliminary evidence that Dap could negatively affect pantothenate synthetase activity *in vitro* (Miyatake *et al.*, 1978). Similarly, growth experiments with *Corynebacterium diphtheriae* demonstrated that Dap antagonized the growth stimulation afforded by β-alanine (Kjerulf-Jensen and Schmidt, 1945). *S. enterica* PanC activity was measured *in vitro* by a coupled assay monitoring the oxidation of NADH (A_{340nm}) in the presence of Dap. Control experiments showed Dap had no effect on the activity of the coupling enzymes. When Dap was added to PanC reactions lacking β-alanine, a background rate of PanC-dependent NADH oxidation was detected (Fig. 3). PanC affinity for Dap (K_m = 8.1 mM) was approximately ten-fold less than for β-alanine (K_m = 0.66 mM; Bazurto and Downs, 2014). These data suggested Dap could interfere with pantothenate production *in vivo*, thereby eliciting a pantothenate requirement when Dap accumulation was provoked.

The cause of detectable PanC activity when Dap, but not β-alanine, was present in the reactions was investigated. It was formally possible that the stock of Dap had contaminating β-alanine or alternatively that Dap was serving as a substrate for PanC, leading to the formation of a pantothenate analog. PanC reactions were set up containing the canonical PanC substrates, or Dap in lieu of β-alanine, and the reaction products were analyzed in each case. The completed reactions, and the corresponding unreacted controls, were derivatized with OPA and separated by high performance liquid chromatography. Comparing HPLC traces between the Dap stock and a β-alanine standard ruled out β-alanine contamination in the Dap stock and indicated that a unique PanC product had been formed when Dap was present (data not shown). The identity of the Dap-dependent PanC product was identified by mass spectrometry. Aliquots from the PanC reactions described above were analyzed by electrospray ionization mass spectrometry in the positive mode after elution from a liquid

chromatography column. The data showed that, as expected, pantothenate was formed in reactions containing β-alanine (m/z = 220; Fig. 4a). In reactions containing Dap instead of β-alanine, no pantothenate was formed and a major chemical species with a mass-to-charge ratio (m/z = 235) that matched the predicted product formed through the condensation of pantoate and Dap was present (Fig. 4b). Together these data established that Dap had the ability to inhibit pantothenate production in *S. enterica*, and suggested that *in vivo*, Dap would be converted to the pantothenate analog, 2-aminopantothenate, at some frequency. The observation that intact DpaL enzyme prevented Dap from causing a pantothenate limitation in *S. enterica* highlighted the role of DpaL in mitigating Dap stress.

RidA quenches 2AA produced by DpaL in vitro

The α,β-elimination reaction catalyzed by DpaL acting on Dap as a substrate was predicted to proceed through a 2AA intermediate, followed by spontaneous conversion to pyruvate (Bisht *et al.*, 2012). This suggested the DpaL enzyme could belong to a growing class of PLP-dependent enzymes that contribute to 2AA stress in the absence of RidA. Addition of RidA to enzymatic reactions that released 2AA into solution led to detectable increases in the rate of 2AA hydrolysis to pyruvate *in vitro* (Lambrecht *et al.*, 2012; Ernst *et al.*, 2014). Recombinant DpaL protein was purified and assayed in the presence and absence of RidA. In the absence of RidA, recombinant DpaL displayed specific activity (4.9 μmol/min/mg) consistent with previous reports (Fig. 5) (Khan *et al.*, 2003). Adding RidA to the reaction at a 3:1 active site ratio of RidA (trimer) to DpaL (dimer) resulted in a ~64 % increase in the rate of pyruvate formation at 6 mM Dap (10.8 nmol/min without RidA versus 17.3 nmol/min with RidA) (Fig. 5). At high concentrations of Dap (≥48 mM), RidA had no effect on the rate of pyruvate formation (Fig. 5). A possible interpretation of these data is that when Dap concentrations are elevated, RidA hydrolase activity becomes inhibited. A physiological significance of this finding is unlikely given the artificially high concentrations of Dap required to inhibit RidA *in vitro*. Nonetheless, this observation differs from the previously observed direct sequestration of 2AA by cysteine (Kredich *et al.*, 1973; Ernst *et al.*, 2014), in that elevated concentrations of Dap only inhibited reactions containing RidA. The generation and release of 2AA by DpaL *in vitro* suggested that this enzyme contributed to 2AA stress *in vivo*.

DpaL influences the sensitivity of a ridA strain to Dap

The potential for DpaL to contribute to 2AA stress in *S. enterica* was tested by growing a *ridA* strain (DM14829) with 0.25 mM Dap. In the presence of Dap a *ridA* mutant had a growth defect that could not be rescued by proline and/or pantothenate (Fig. 6). To clarify the cause of the growth defect, a *ridA dpaL* double mutant strain (DM14882) was constructed and tested under appropriate growth conditions. Disrupting *dpaL* in a *ridA* background eliminated Dap sensitivity of the *ridA* strain when proline and pantothenate were included in the growth medium (Fig. 6). When compared to data presented in Figure 2, it was apparent that growth of the *ridA dpaL* mutant was not restored to the same level as the *dpaL* single mutant despite proline and pantothenate supplementation, implicating additional nutrient limitation(s) caused by the *ridA* mutation. Isoleucine, added in combination with proline and pantothenate, restored full growth of the *ridA dpaL* strain (Fig. 6). Importantly, growth of the *ridA* strain (DM14829) was not restored wherever Dap was added to the

growth medium. These data were consistent with a model wherein DpaL degrades Dap and releases 2AA into the cellular milieu, leading to 2AA stress and growth inhibition in the absence of RidA. This scenario was supported by the finding that the addition of glycine partially alleviated the Dap sensitivity of a *ridA* strain (data not shown). Glycine relieves the growth inhibition caused by 2AA damage of serine hydroxymethyltransferase (GlyA; EC 2.1.2.1) in strains lacking RidA (Flynn *et al.*, 2013; Ernst and Downs, 2015).

Previous work showed isoleucine biosynthesis is negatively impacted by 2AA produced from endogenous serine in the absence of *ridA* (Schmitz and Downs, 2004; Christopherson *et al.*, 2008; Lambrecht *et al.*, 2013). Endogenously generated 2AA inhibited the final step of the isoleucine pathway catalyzed by transaminase B (IlvE; EC 2.6.1.42), and reduced flux to isoleucine (Schmitz and Downs, 2004; Christopherson *et al.*, 2008). It was possible that a combined effect of 2AA (from serine) and Dap on the isoleucine biosynthetic pathway explained the stimulatory effect of isoleucine on growth of the *ridA dpaL* (DM14882) strain in the presence of Dap. Biosynthetic threonine dehydratase (IlvA) catalyzes the first committed step in isoleucine biosynthesis and is susceptible to inhibition by amino acids structurally related to threonine, including serine (Harris, 1981; Burns *et al.*, 1979). When monitored *in vitro*, Dap displayed mixed inhibition of IlvA with respect to threonine (K_i = 1.7 mM) (Fig. 7). The conversion of Dap to pyruvate by IlvA could not be detected under the conditions tested (data not shown). The mixed nature of inhibition is consistent with the ability of amino acids to bind to allosteric sites on the enzyme while simultaneously competing with threonine substrate for access to the IlvA active site (Burns *et al.*, 1979). *In vivo* data demonstrated a role for RidA in preventing metabolic stress caused by 2AA generated from the degradation of Dap mediated by DpaL. Abolishing DpaL in a *ridA* strain alleviated 2AA stress caused by Dap, but uncovered a minor isoleucine requirement likely stemming from the combined inhibition of IlvA by Dap and damage to the isoleucine pathway caused by Dap-independent 2AA production.

Conclusions

Results presented in this report shed light on the mechanisms of Dap toxicity in *S. enterica* and demonstrated the importance of DpaL and RidA in mitigating sensitivity to Dap (Fig. 8). *In vivo*, Dap elicited a proline requirement in wild type *S. enterica* despite the presence of an intact DpaL enzyme. In the absence of DpaL, Dap accumulation led to the inhibition of pantothenate synthetase, causing a pantothenate limitation. Lastly, the inhibition of threonine dehydratase by Dap contributed to a detectable isoleucine deficiency in the absence of both DpaL and RidA.

These insights raise questions as to whether or not Dap inhibits similar metabolic processes in other organisms. Enteric bacteria including *E. coli* and *S. enterica* maintain low levels of intracellular proline and are unable to induce proline biosynthesis in response to osmotic stress, instead relying on uptake from the extracellular environment (Csonka, 1989). Therefore, enteric bacteria may be particularly susceptible to disrupted proline biosynthesis caused by Dap in the absence of an abundant extracellular supply of proline. *Staphylococcus aureus* is known to produce Dap as a key intermediate in siderophore biosynthesis when grown in the presence of mammalian serum (Kobylarz *et al.*, 2014), yet displays a

conditional proline auxotrophy when grown under these conditions despite the presence of all genes necessary for proline production in the genome (Li *et al.*, 2010). Perhaps our findings highlight the need to temporally coordinate Dap and proline production to prevent inhibitory cross-talk, thereby favoring iron acquisition (i.e. Dap production) over proline production in the context of infection. The finding that Dap specifically inhibits pyrroline 5-carboxylate reductase (ProC) may in part explain the presence of alternative routes to proline in *S. aureus* speculated to be induced under low-iron conditions (Li *et al.*, 2010), where Dap production would likely be elevated.

The conserved function of pantothenate synthetase among bacteria, plants and fungi suggests that organisms that rely on *de novo* coenzyme A production may be sensitive to excessive Dap accumulation. *Mycobacterium tuberculosis* was attenuated when PanC activity was disrupted, confirming that *de novo* pantothenate biosynthesis is important for the pathogenic lifestyle of some organisms (Kumar *et al.*, 2013; Devi *et al.*, 2014). Furthermore, these findings may inform future drug discovery efforts, as the majority of pantothenate synthetase inhibitors described to date have been analogues of ATP or pantoate (Tuck *et al.*, 2006). Although Dap serves as a precursor to many secondary metabolites and antibiotics, data presented here and elsewhere (Kjerulf-Jensen and Schmidt, 1945; Garcia-Hernandez and Kun, 1957) show that Dap itself elicits antimicrobial effects and may influence microbial community dynamics in the environment. The potential toxicity of Dap suggests that organisms that produce Dap endogenously likely regulate Dap concentrations in the cell, or have developed alternative metabolic strategies to circumvent nutrient limitations caused by Dap.

Diaminopropionate ammonia-lyase represents an additional generator of endogenous 2AA in *Salmonella*. These results confirm a pattern of 2AA generation mediated by fold-type II PLP-dependent enzymes acting on amino acid substrates. Homologs of DpaL have not been observed in any eukaryotic organisms to date, suggesting the dedicated capacity to degrade Dap is likely unique to prokaryotes (Bisht *et al.*, 2012; Kalyani *et al.*, 2012). However, many fold-type II PLP enzymes are found throughout each domain of life, suggesting that several mechanisms of endogenous 2AA production likely exist. This report corroborates previous findings related to cysteine detoxification by CdsH, showing that RidA participates in amino acid detoxification by preventing concomitant 2AA stress. These findings also raise broader questions regarding the significance of 2AA. *S. enterica* degrades toxic levels of serine by an Fe-S dependent mechanism catalyzed by serine dehydratase (SdaA; EC 4.3.1.17). Our unpublished work and a recent report confirmed that the SdaA enzyme does not release a 2AA substrate for RidA (Xu and Grant, 2016). In light of alternative options, why employ a mechanism that releases potentially damaging 2AA? Perhaps RidA hydrolase activity confers a selective advantage to PLP-dependent 2AA release mechanisms that are less susceptible to oxidative damage in comparison to Fe-S dependent mechanisms. Alternatively, free 2AA may interact with cellular components in order to serve as an important regulatory signal akin to hydrogen peroxide (Rhee, 2006). Regardless, the frequent co-occurrence of PLP-dependent eliminases and RidA homologs among distantly related organisms supports the argument for an evolutionarily conserved role of RidA in preventing 2AA stress (Niehaus *et al.*, 2015).

Experimental procedures

Bacterial strains, media and chemicals

The bacterial strains used in this study were generated from *Salmonella enterica* serovar Typhimurium LT2. Tn*10d* indicates the transposition-defective mini-Tn*10* (Tn*10*Δ16Δ17) described previously (Way *et al.*, 1984). Minimal medium consisted of no-carbon E medium (NCE) supplemented with 1 mM $MgSO_4$ (Castilho *et al.*, 1984), trace minerals (Balch and Wolfe, 1976) and 11 mM D-glucose as the sole carbon source. Standard rich medium was Difco nutrient broth (8 g/liter) with sodium chloride (5 g/liter). Superbroth containing tryptone (32 g/liter), yeast extract (20 g/liter), sodium chloride (5 g/liter) and sodium hydroxide (0.2 g/liter) was used when high cell densities were required. Solid medium was made by adding Difco BiTek agar (15 g/liter). Antibiotics were added as needed to minimal and rich growth medium at the following concentrations, respectively: tetracycline, 10 and 20 μg/ml; kanamycin, 12.5 and 50 μg/ml; chloramphenicol, 5 and 20 μg/ml; ampicillin, 15 and 150 μg/ml. L-2,3-diaminopropionate was purchased from Chem-Impex International, Inc., Wood Dale, IL. Amino acids and vitamins used in growth and enzyme activity assays were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO.

Genetic techniques and growth analysis

Genetic crosses were performed by transduction using the high-frequency general transducing mutant of bacteriophage P22 (HT105/1, *int*-201) (Schmieger, 1972). Standard methods used for performing transductions, purifying transductants from phage and identifying phage-free recombinants were described previously (Chan *et al.*, 1972; Downs and Petersen, 1994). The gene replacement made in *STM1002 (dpaL)* was constructed using the λ-Red recombinase system described by Datsenko and Wanner (Datsenko and Wanner, 2000). To be consistent with the described activity of diaminopropionate ammonia-lyase, the *STM1002* gene locus was renamed *dpaL*.

Growth phenotypes were established in solid medium by using agar overlays. Concentrated amino acid or vitamin stocks were spotted (5 μl) on the overlay surface and plates were incubated overnight at 37°C. Growth phenotypes were determined in liquid medium by growing cultures to full density in NB at 37°C. Cells were then pelleted and resuspended in an equal volume of saline solution (85 mM), and 2 μl of the resuspension was inoculated into 198 μl of the relevant defined growth medium contained in a 96-well microtiter plates. Microtiter plates were incubated at 37°C while shaking in a Biotek EL808 ultra microplate reader. Growth was measured as the change in absorbance at 650 nm (OD_{650}) over time. Data were plotted as the average and standard deviation of three biological replicates using GraphPad Prism 5.0f with the Y-axis displayed in $\log_{10}$ format.

Proline cross-feeding experiments were performed in agar overlays. Reporter strains (proline auxotrophs) were grown overnight in NB liquid at 37°C, washed once with NaCl and overlaid in soft-agar on solid minimal glucose medium containing 1.3 mM L-arabinose (Sigma). L-diaminopropionate was included in the growth medium at a final concentration of 0.25 mM, where appropriate. Producer strains were freshly streaked for isolation and grown overnight at 37°C on NB medium containing ampicillin. Single colonies were picked

and stabbed into the solid overlay of the reporter strain and incubated at 37°C for 48 hours. Growth of the reporter strain was assessed based on the change in turbidity in the embedded agar overlay surrounding the stab site. Growth of the producer strain was determined based on outgrowth from the surface of the inoculation site and was clearly distinguishable from growth of the embedded reporter strain.

Molecular methods

The *dpaL* gene was amplified by PCR using Q5 High-fidelity DNA polymerase (New England BioLabs) with primers, DpaL-for-NdeI (5'-AGAGAGCATATGCATGAGCTTAT-3') and DpaL-rev-BamHI (5'-AGAGAGGGATCCTTAAGCACT-3'). The resulting PCR product was gel purified, digested with NdeI (New England BioLabs) and BamHI (New England BioLabs), and ligated into pET-15b (Novagen) vector digested with the same restriction enzymes, forming pDM1451. Constructs were transformed into *Escherichia coli* strain DH5α and the resulting transformants were screened for vectors containing the appropriate insert. Plasmid inserts were confirmed by sequence analysis, performed by Genewiz.

The *proB* and *proA* genes from *S. enterica* were amplified by PCR as a single amplicon using GoTaq Green Master Mix (Promega) with primers, proAB_NcoI_F (5'-GAGACCATGGCCATGAGTGACAGCCAGACG −3') and proAB_PstI_R (5'-GAGACTGCAGTTACGCACGAATCGTACC −3'). The PCR reaction was cleaned-up, digested with NcoI (New England BioLabs) and PstI (New England BioLabs), gel purified and ligated into pBAD24 (Guzman *et al.*, 1995) previously digested with the same restriction enzymes, forming pDM1457. Constructs were transformed into DH5α and the resulting transformants were screened by PCR for the appropriate insert and confirmed by sequence analysis through Eton Bioscience Inc. The *proB* gene encoded by pDM1457 was altered by site-directed mutagenic PCR using the primer proAB_D107N (5'-CGGGCAGATGCTGTTGACGCGTGCGAATATGGAAGACAGAGAGCGCTTTCTG −3') and *PfuUltra* High-Fidelity DNA polymerase (Agilent), forming pDM1462. As a result, basepair 319 of *proB* was changed from G-to-A, altering the encoded protein to $ProB^{D107N}$, consistent with a previously reported feedback-resistant variant of ProB from *E. coli* (Dandekar and Uratsu, 1988). The $proB^{G319A}$ allele generated in pDM1462 was confirmed by sequence analysis, performed by Eton Bioscience Inc.

Purification of diaminopropionate ammonia-lyase (DpaL)

Wild type *dpaL* cloned into pET-15b (pDM1451), containing an N-terminal polyhistidine-tag, was transformed into *E. coli* BL21-AI for overexpression and Ni-affinity purification. Cells were inoculated into 10 ml of superbroth containing ampicillin and grown overnight at 37°C. Overnight cultures were sub-cultured into 3 liters of superbroth containing ampicillin and grown at 37°C while shaking at 250 rpm until an OD_{650} of 0.5 was reached. Expression was induced by adding arabinose (0.2 %) and IPTG (0.1 mM) and cultures were shifted to 30°C for 16 hours. Cells were pelleted at 4°C by centrifugation (15 min at 8,000 x *g*) and resuspended in buffer A (50 mM potassium phosphate pH 7.5 containing 100 mM sodium chloride, 5 mM imidazole and 10 % glycerol). Lysozyme (30 mg) and DNase (1 mg) were added to the cell suspension and incubated on ice for 30 minutes. Cells were mechanically

lysed using a French pressure cell (3 passes at 10,342 kPa). The resulting crude cell lysate was clarified by centrifugation (1 hr at 48,000 x *g*) and filtered through a 0.22-μm polyethersulfone membrane (Whatman). The filtered lysate was added to a column containing 5 ml of Ni-nitriloacetic acid (NTA) Superflow resin (Qiagen). The column was washed with 50 ml of buffer A, then eluted with buffer B (buffer A + 500 mM imidazole) added to the column in a linear gradient over 10 column volumes. The resulting fractions were analyzed for purity, then pooled and dialyzed against 2 × 1 L of 20 mM Tris-HCl pH 8 containing 100 mM NaCl for 3 hrs, followed by 1 × 1 L of 20 mM Tris-HCl pH 8 containing 5 % glycerol overnight. Protein recovery as determined by the BCA assay (Pierce) was 4.2 mg/ml. Protein aliquots were frozen in liquid nitrogen and stored at −80°C.

Diaminopropionate ammonia-lyase (DpaL) assays

Diaminopropionate ammonia-lyase (DpaL) activity was measured by coupling pyruvate formation to NADH oxidation using lactate dehydrogenase (Sigma) and monitoring the decrease in absorbance at 340 nm as previously described (Kalyani *et al.*, 2013). RidA was purified and its activity was confirmed previously (Ernst *et al.*, 2014). Assays consisted of 100 mM Tris-HCl pH 8, 0.25 mM NADH, and 5 U lactate dehydrogenase. DpaL and RidA were added at final monomeric concentrations of 0.2 μM and 0.9 μM, respectively, achieving a 1:3 ratio of oligomeric DpaL (dimer) to RidA (trimer). L-2,3-diaminopropionate was added at final concentrations ranging from 0 – 80 mM to initiate reactions. Each reaction consisted of 300 μl and was monitored continuously at 22°C in a 96-well quartz plate using a SpectraMax M2 (Molecular Devices) microplate reader. Initial rates of NADH consumption were calculated from the linear change in absorbance at 340 nm over the course of 2 minutes following an initial non-linear phase (~60 seconds). A standard curve of NADH concentration based on A_{340} was used to calculate enzyme activity, reported as the initial rate of pyruvate formation in μmol/min. Experiments were performed in triplicate, and the resulting data were plotted using GraphPad Prism 5.0f and fit to the Michaelis-Menten model where appropriate.

Purification of γ-glutamate kinase (ProB) and glutamyl 5-semialdehyde dehydrogenase (ProA)

ProB and ProA, encoded by the respective genes from *E. coli*, were purified from ASKA strains possessing the appropriate pCA24N constructs (Kitagawa *et al.*, 2006). The resulting proteins contained N-terminal hexahistidine tags. Strains were grown overnight at 37°C in 10 ml of superbroth containing chloramphenicol. Overnight cultures were inoculated into 1 L each of superbroth containing chloramphenicol and cultures were grown at 37°C with aeration until an OD_{650} of 0.5 was reached. Expression was induced by adding IPTG (0.02 %) and cultures were shifted to the appropriate growth conditions. Expression of *proB* was performed at 37°C for 8 hours. Expression of *proA* was carried out at 20°C overnight (~16 hours). Cells were then harvested by centrifugation (15 min at 8,000 x *g*) and frozen at −80°C overnight. Cell pellets were thawed and resuspended in 50 mM Tris-HCl pH 7.2 containing 200 mM NaCl, 1 mM TCEP and 10 mM imidazole. Lysozyme (10 mg), DNase (0.25 mg) and phenylmethanesulfonyl fluoride (1 mg) were added and the cell suspensions were incubated on ice for 45 minutes. Cells were mechanically lysed using a French pressure cell (3 passes at 10,342 kPa). The resulting lysates were clarified by centrifugation

and passed through 0.45-μm polyethersulfone filters (Whatman). A 50 % slurry of Ni-NTA Superflow (Qiagen) resin was added (2 ml) to each lysate and incubated at 4°C with gentle shaking for 1 hour. The resin-lysate mixes were added to 10 ml Poly-Prep (Bio-Rad) chromatography columns and allowed to settle. Each column was washed with 30 ml of 50 mM Tris-HCl pH 7.2 containing 200 mM NaCl, 1 mM TCEP, 40 mM imidazole and 10 % glycerol. ProB and ProA were then eluted from each column by adding 5 ml of elution buffer (50 mM Tris-HCl pH 7.2 containing 200 mM NaCl, 1 mM TCEP, 300 mM imidazole and 10 % glycerol). Fractions (0.5 ml) were collected and pooled together following SDS-PAGE analysis to determine protein purity. The buffer was exchanged with 50 mM Tris-HCl pH 7.2 containing 10 % glycerol using a PD-10 Sephadex G25 column (GE Healthcare). The concentration of ProB and ProA protein recovered as determined by the BCA assay (Pierce) was 1.2 mg/ml and 8.1 mg/ml, respectively. Protein aliquots were frozen in liquid nitrogen and stored at −80°C until use.

γ-glutamate kinase (ProB) and glutamyl 5-semialdehyde dehydrogenase (ProA) assays

Continuous assays—ProB and ProA were assayed together by measuring the rate of NADPH oxidation catalyzed by ProA, with the labile substrate (γ-glutamyl phosphate) generated from glutamate by ProB, as previously described (Perez-Arellano *et al.*, 2004). Assays consisted of 100 mM Tris-HCl pH 7.2 containing 16 mM $MgCl_2$, 4 mM ATP, 0.3 mM NADPH, 0.2 μM ProB and 0.6 μM ProA. L-2,3-diaminopropionate was added at various concentrations (0 – 10 mM) to determine the impact on ProAB activity. Reactions (300 μl) were initiated by adding L-glutamate (12 mM) and the change in A_{340} corresponding to NADPH oxidation was monitored for 5 minutes. Experiments were performed in a 96-well quartz plate using a SpectraMax M2 (Molecular Devices) microplate reader. Initial rates were calculated based on the extinction coefficient of NADPH (ε = 6,220 M^{-1} cm^{-1}), and were reported in μmol/min. Representative data are presented in the text as the average and standard deviation of three independent experiments.

End-point assays—ProAB endpoint assays were completed in 0.5 ml reaction volumes consisting of 50 mM MES/HEPES/TAPS pH 7.5 containing 16 mM $MgCl_2$, 4 mM ATP, 1 mM NADPH, 0.4 μM ProB and 2 μM ProA. NADPH was excluded in a subset of reactions intended to accumulate γ-glutamyl phosphate. L-2,3-diaminopropionate (10 mM) was included when appropriate and L-glutamate (12 mM) was added to initiate reactions, followed by incubation at 30°C for 16 hours. Protein was removed using a 10,000 Da molecular weight cutoff microcentrifuge filter (Millipore). Aliquots (20 μl) of the resulting protein-free solution were derivatized with *o*-phthalaldehyde (OPA; 80 μl) for 90 seconds and directly injected onto a Gemini C18 column (Phenomenex) pre-equilibrated with methanol-tetrahydrofuran-50 mM sodium acetate buffer pH 6.6 (24:1:75). The concentration of methanol was increased in a linear gradient to 80% over the course of 20 minutes while absorbance (λ = 190 – 600 nm) and fluorescence (Ex: 340 nm; Em: 455 nm) were monitored in tandem using the SPD-M20A (Shimadzu) and RF-20Axs (Shimadzu) detectors, respectively. Peaks corresponding to all known reaction components were accounted for. In an alternative approach to derivatization and HPLC analysis, protein-free reaction aliquots were submitted directly for LC/MSD-TOF analysis at the University of

Wisconsin Biotechnology Center (Madison, WI) and were analyzed in the positive mode following reverse phase separation.

Pantothenate synthetase (PanC) assays

PanC was provided by J. V. Bazurto and was purified as previously described (Bazurto and Downs, 2014).

Continuous assays—PanC assays were performed using a coupled enzyme system according to previous reports (Tuck *et al.*, 2006; Bazurto and Downs, 2014). Briefly, the ATP-dependent ligation of pantoate to β-alanine produced AMP. AMP and ATP were converted to 2 moles of ADP by myokinase. The resulting ADP was used to accept a phosphate from phosphoenolpyruvate in a reaction catalyzed by pyruvate kinase, generating pyruvate. Finally, lactate dehydrogenase catalyzed the oxidation of NADH to reduce pyruvate to lactate.

Reactions (200 μl) consisted of 100 mM Tris-HCl pH 7.5 containing 10 mM $MgCl_2$, 0.3 mM NADH, 1.5 mM phosphoenolpyruvate, 2.5 mM ATP, 5 U myokinase, 5 U pyruvate kinase, 5 U lactate dehydrogenase, 4 mM pantoate and 0.05 μM PanC. L-2,3-diaminopropionate and/or β-alanine were added at increasing concentrations to initiate reactions. Assays were performed in a 96-well quartz plate and monitored for 5 minutes using a SpectraMax M2 (Molecular Devices) microplate reader. Initial rates were calculated from the change in A_{340} corresponding to NADH oxidation. Based on the coupling assay design, 1 mol of pantothenate (or pantothenate analog) was produced per 2 moles of NADH oxidized. A standard curve of A_{340} plotted as a function of NADH concentration was used to calculate rates, reported as nmol NADH consumed per min. The resulting data were fit with a curve using the Michaelis-Menten equation in GraphPad Prism 5.0f.

Endpoint assays—PanC endpoint assays were performed in 0.5 mls and consisted of 50 mM potassium phosphate buffer pH 8 containing 10 mM $MgCl_2$, 2.5 mM ATP, 4 mM pantoate and 1 μM PanC. L-2,3-diaminopropionate (5 mM) was added when appropriate. Reactions were initiated by adding β-alanine and assays were incubated at 28°C for 16 hours. PanC was removed from the reactions using a 30,000 Da molecular weight cutoff centrifuge filter (Millipore). Aliquots (40 μl) of the resulting protein-free solution were mixed with OPA (80 μl) and allowed to react for 90 seconds at room temperature, while the remainder of the sample was frozen at −20°C. Derivatized samples were then analyzed by reverse-phase high-performance liquid chromatography using the Shimadzu Prominence HPLC system. Absorbance and fluorescence were monitored in tandem by using the SPD-M20A photo diode array detector (λ = 190 – 600 nm) and the RF-20Axs fluorescence detector (Ex: 340 nm; Em: 455 nm), respectively. A Gemini C18 column (Phenomenex) equipped with a C18 SecurityGuard (Phenomenex) cartridge was pre-equilibrated with 60 % mobile phase A (water containing 0.1 % trifluoroacetic acid pH 2.5) and 40 % mobile phase B (methanol containing 0.1 % trifluoroacetic acid pH 2.5). Samples (20 μl) were introduced onto the column at a flowrate of 1 ml/min and eluted by increasing the concentration of mobile phase B to 100 % over 20 minutes. Samples displaying unique product peaks indicative of Dap-derivatization were identified for follow-up analysis by mass spectrometry

(MS). The corresponding non-derivatized samples and appropriate controls were submitted for MS analysis at the University of Wisconsin Biotechnology Center (Madison, WI). Crude samples (5 μl) were pre-separated by liquid chromatography using a Zorbax SB-C18 column (Agilent) and mass data were obtained by MSD-TOF analyzed in the positive mode.

Threonine dehydratase (IlvA) assays

IlvA was provided by K. Hodge-Hanson and was purified following a previously described protocol (Lambrecht *et al.*, 2012). Reactions (300 μl) consisted of 50 mM potassium phosphate pH 8 and 65 nM IlvA. L-2,3-diaminopropionate was added at increasing concentrations. Experiments were performed in triplicate and measured in a 96-well quartz plate using a SpectraMax M2 (Molecular Devices) microplate reader. Reactions were initiated by adding L-threonine and monitored continuously at 230 nm for 60 seconds. Initial rates were determined based on the increase in A_{230} corresponding to α-ketobutyrate (AKB) production. A standard curve of AKB concentrations relative to A_{230} was generated and used to calculate reaction rates, reported as μmol of AKB produced per minute. The data, reported as the average and standard deviation of three independent experiments, were fit with curves corresponding to a mixed model of inhibition using GraphPad Prism 5.0f. Lineweaver-Burke plots were generated and used to confirm the mode of inhibition.

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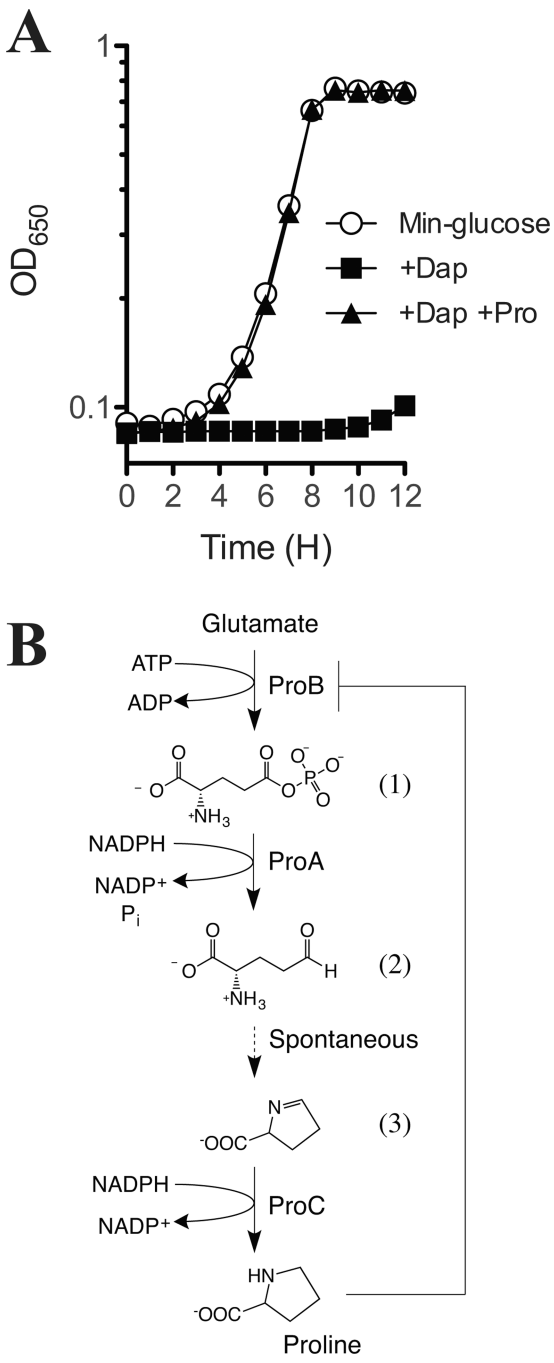

Fig. 1.

L-2,3-diaminopropionate elicits a proline requirement in *S. enterica*. A) Wild type *S. enterica* (DM14828) was grown at 37°C in glucose minimal medium containing no addition (○); 0.25 mM Dap (■); or 0.25 mM Dap and 1 mM proline (▲). The data reflect experiments performed in triplicate. Error bars representing the standard deviation are not visible because the replicates deviated less than 5 % from the mean. B) Proline biosynthesis in *S. enterica* occurs in four steps, beginning with the ATP-dependent phosphorylation of glutamate, generating γ-glutamyl 5-phosphate (1). The conversion of glutamate 5-

semialdehyde (2) to 1-pyrroline-5-carboxylate (3) occurs spontaneously (dashed line). Proline feedback-regulates the first enzyme (ProB) in the pathway.

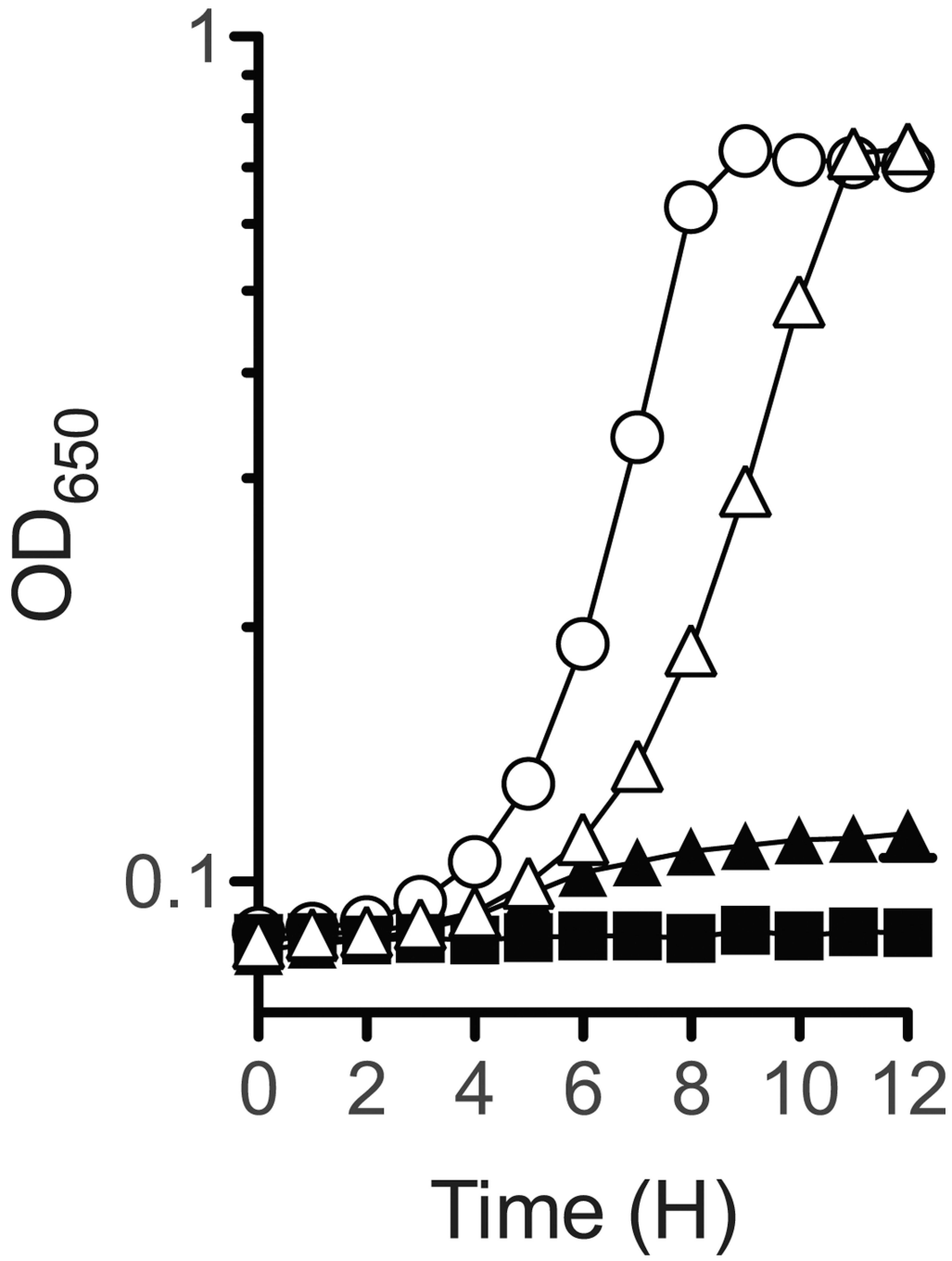

Fig. 2.

Pantothenate and proline are required for growth of a *dpaL* mutant in the presence of Dap. A *dpaL* mutant strain (DM14881) was grown at 37°C in glucose minimal medium containing no addition (○); 0.25 mM Dap (■); 0.25 mM Dap and 1 mM proline (▲); or 0.25 mM Dap, 1 mM proline, and 0.1 mM pantothenate (Δ). The data reflect experiments performed in triplicate. Error bars representing the standard deviation are not visible as the replicates deviated less than 5 % from the mean. Growth with Dap and pantothenate together was indistinguishable from Dap alone under the conditions tested (data not shown).

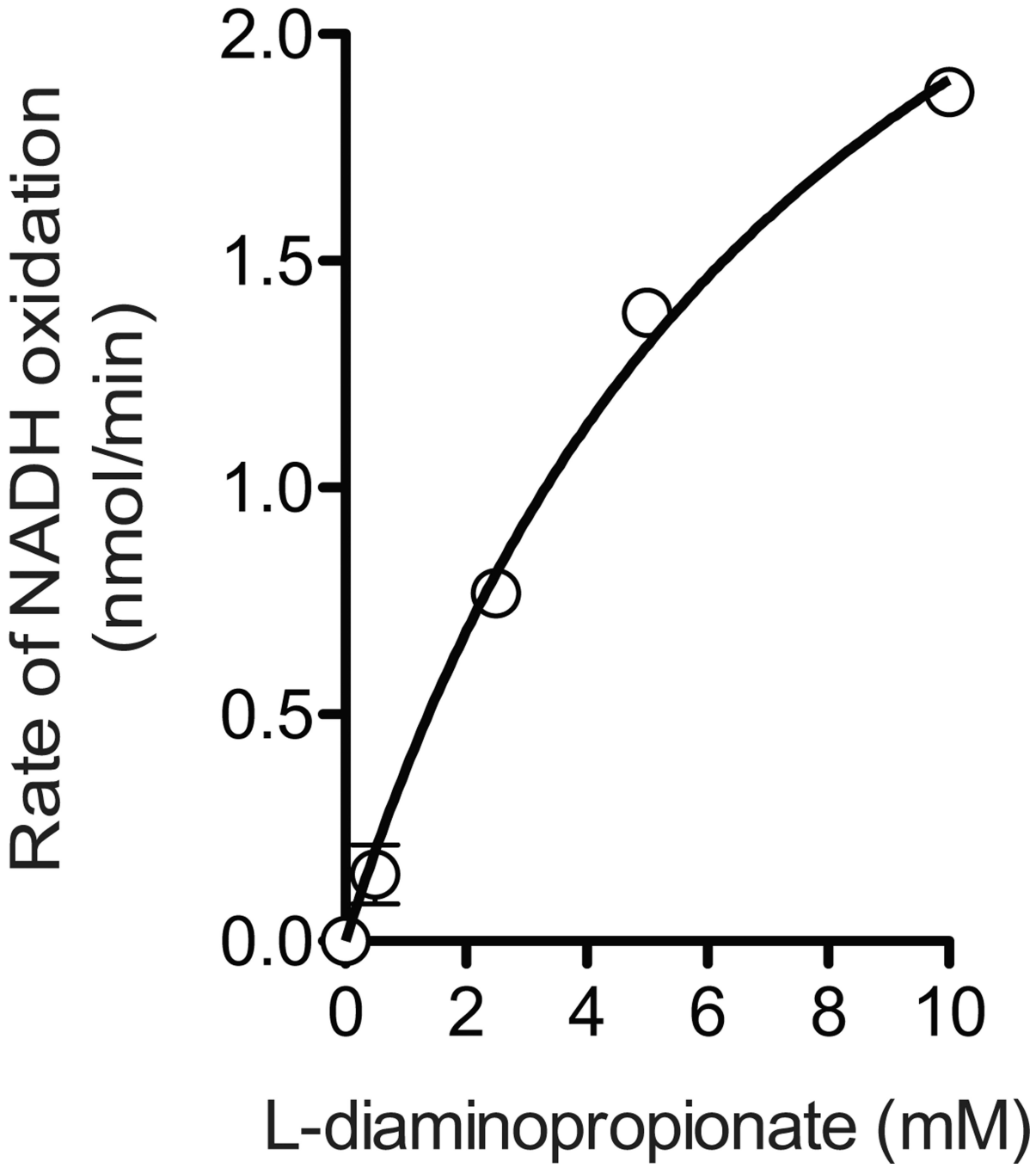

Fig. 3.

L-2,3-diaminopropionate serves as a substrate for pantothenate synthetase. PanC activity was measured by a coupled assay, relying on the oxidation of NADH (ΔA_{340nm}), in the presence of increasing concentrations of Dap. The initial rate of NADH oxidation in the presence of Dap was determined in the absence of β-alanine. Observed rates were completely dependent on the addition of enzymes and co-substrates as outlined in *Experimental procedures*. Data are plotted as the average and standard deviation of three

independent experiments. The data were fit with a curve in GraphPad Prism 5.0f using the Michaelis-Menten equation.

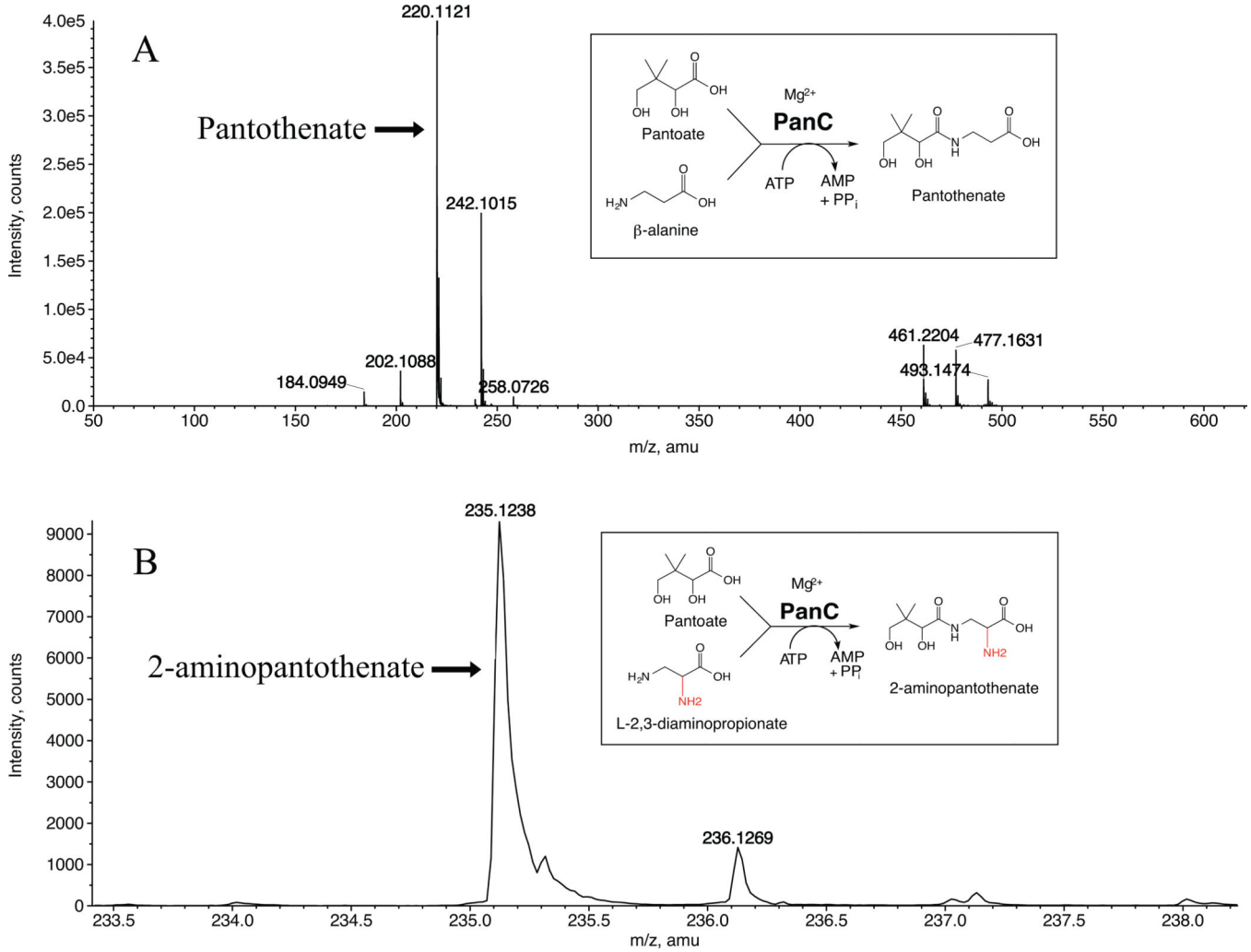

Fig. 4.

L-2,3-diaminopropionate reacts with pantoate in the presence of PanC to form a pantothenate analog. PanC assays containing pantoate, ATP and A) β-alanine or B) L-2,3-diaminopropionate as substrates were carried out for 16 hours. PanC was removed by size-exclusion filtration and samples were submitted for LC/MSD-TOF analysis in the positive mode. Insets show the reaction components and anticipated products, pantothenate (219 g/mol; m/z = 220) and 2-aminopantothenate (234 g/mol; m/z = 235), respectively.

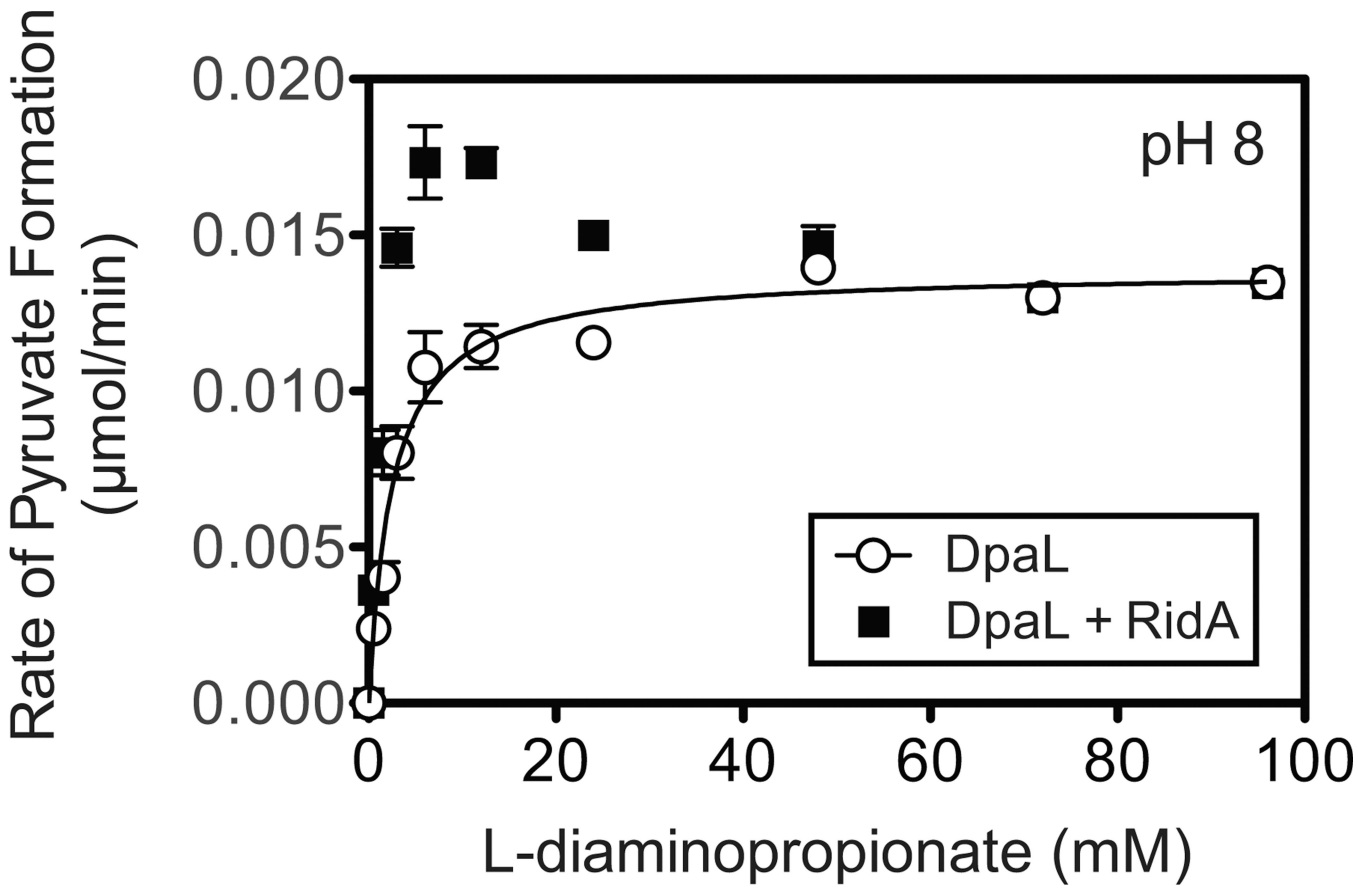

Fig. 5.

Diaminopropionate ammonia-lyase (DpaL) generates a 2AA substrate for RidA *in vitro*. Reactions consisted of DpaL (0.2 µM) alone (○) or DpaL and RidA (0.9 µM) (■). Assays were performed in triplicate and are plotted as the average and standard deviation. The data from DpaL-only assays were fit with a curve in GraphPad Prism 5.0f using the Michaelis-Menten equation.

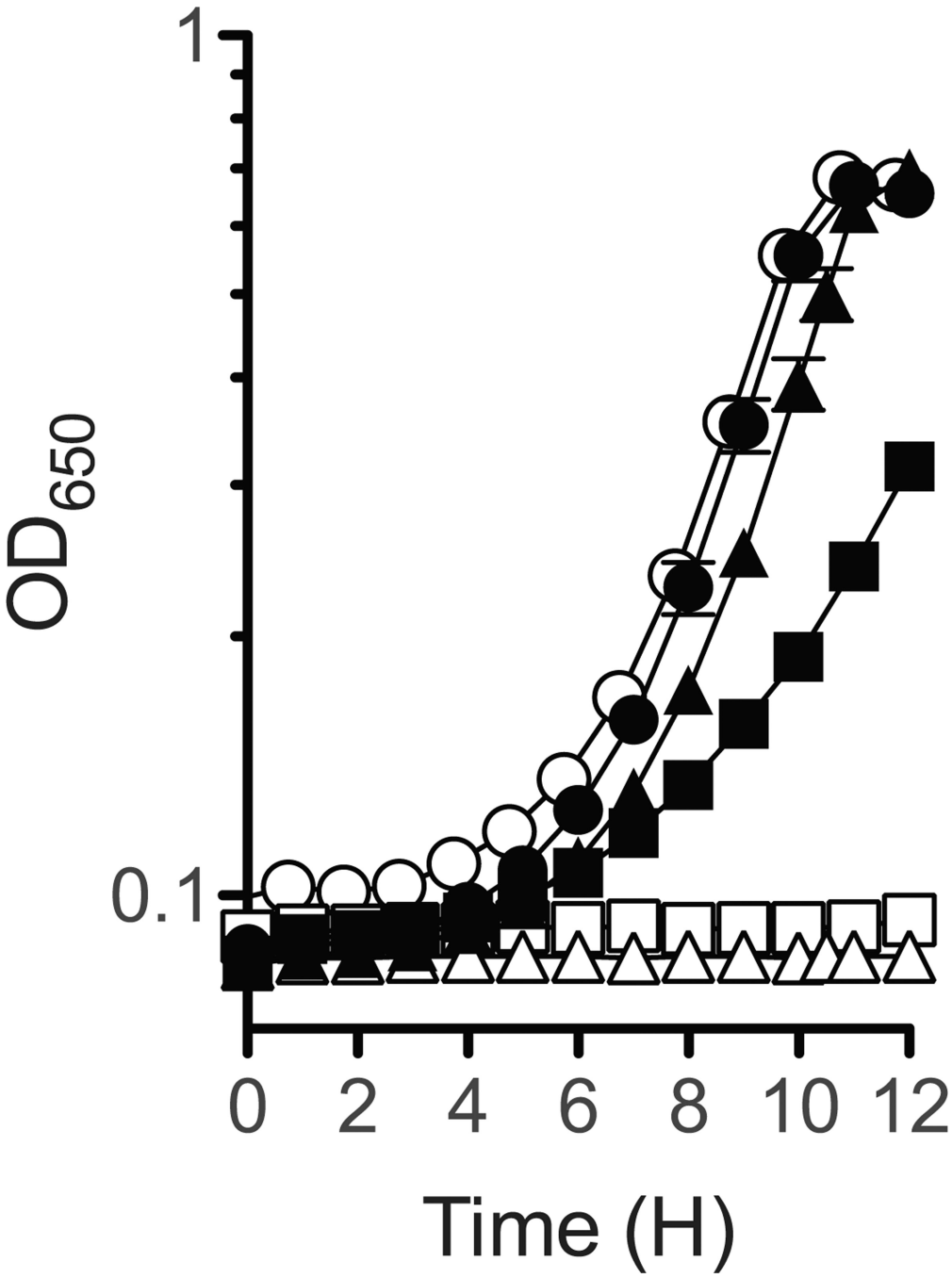

Fig. 6.

DpaL influences the sensitivity of a *ridA* strain to inhibition by Dap. A *ridA* strain (DM14829, open symbols) and a *ridA dpaL* strain (DM14882, closed symbols) were grown in glucose minimal medium containing no addition (circles); 0.25 mM Dap, 1 mM proline , and 0.1 mM pantothenate (squares); or 0.25 mM Dap, 1 mM proline , 0.1 mM pantothenate, and 1 mM L-isoleucine (triangles). Experiments were performed in triplicate and plotted as the average and standard deviation.

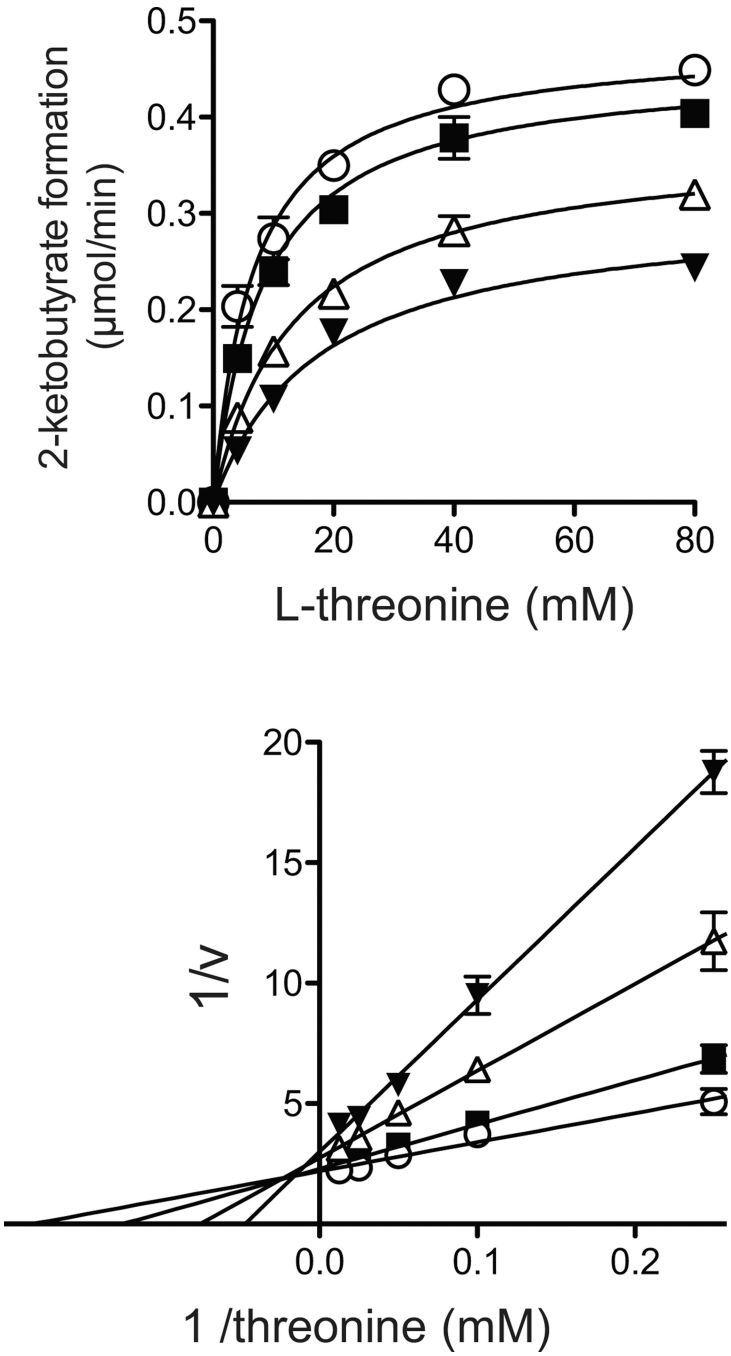

Fig. 7.

L-2,3-diaminopropionate inhibits IlvA. IlvA assays were performed in the presence of varying concentrations of Dap: 0 mM (○; R^2 = 0.97), 0.5 mM (■; R^2 = 0.98), 2.5 mM (Δ; R^2 = 0.97), and 5 mM (▼; R^2 = 0.98). Data are plotted as the average and standard deviation of three independent experiments performed in parallel. (Top) The data were fit according to a mixed model of inhibition using GraphPad Prism 5.0f. (Bottom) Lineweaver-Burke analysis demonstrates mixed inhibition.

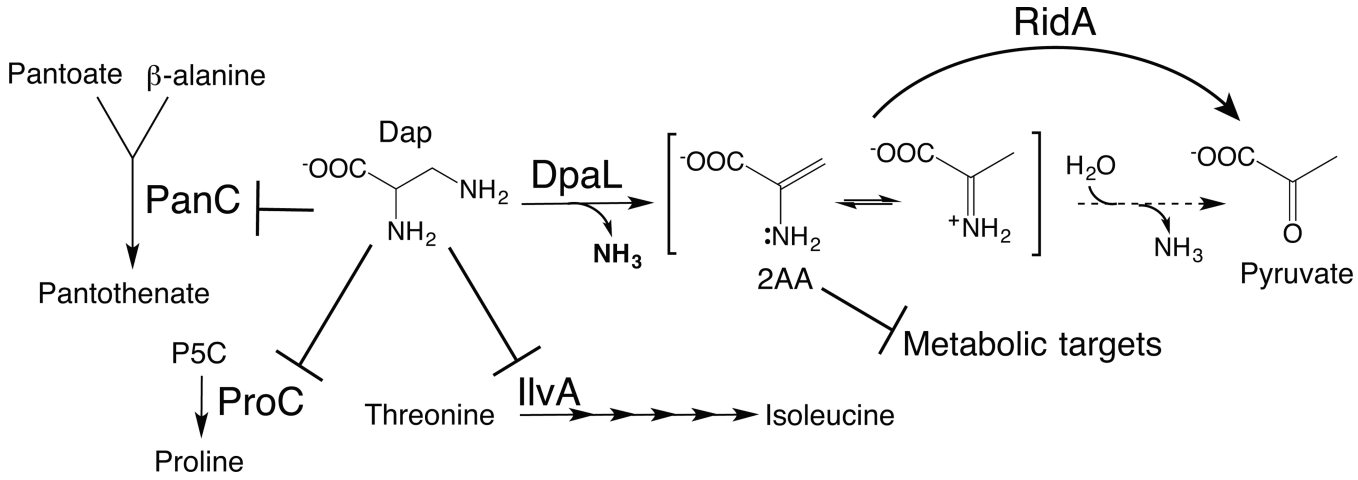

Fig. 8.

Proposed role of L-2,3-diaminopropionate (Dap) in the metabolic network. Dap negatively impacts metabolism in *S. enterica* by directly inhibiting proline, pantothenate and isoleucine biosynthesis. Dap also serves as a precursor to 2-aminoacrylate (2AA), leading to 2AA stress in the absence of RidA.

Table 1

Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source
Strains		
TT9667	*proC*693::MudA	Lab collection
DM5846	*proAB::Tn*10d(Tc)	Lab collection
DM14224	*E. coli* BL21-AI / pDM1451	This study
DM14828	Wild type LT2	This study
DM14829	*ridA*1::Tn*10*d(Tc)	This study
DM14881	*dpaL*::Kn	This study
DM14882	*ridA*1::Tn*10*d(Tc) *dpaL*::Kn	This study
DM15352	Wt / pDM1462	This study
DM15353	Wt / pBAD24	This study
DM15356	*proC*693::MudA / pDM1462	This study
DM15357	*proC*693::MudA / pBAD24	This study
Plasmids		
pDM1451	pET15b - $DpaL^{Wt}$	This study
pDM1457	pBAD24 - $ProAB^{Wt}$	This study
pDM1462	pBAD24 - $ProAB^{D107N}$	This study

Tn*10*d(Tc) indicates a transposition-defective mini-Tn*10* (Way *et al.*, 1984). MudA indicates a transposition defective Mu element (Hughes and Roth, 1984). Plasmids constructed using pET15b (Novagen) and pBAD24 (Guzman *et al.*, 1995) are described in the *Experimental procedures*.

Table 2

Dap inhibits ProC *in vivo*

Reporter strain[a]	Media[b]	Producer strain[c]	
		Wt/pDM1462 (DM15352)	*proC*/pDM1462 (DM15356)
proAB (DM5846)	− Dap	+[d]	+
	+ Dap	+	−
proC (TT9667)	− Dap	+	−
	+ Dap	+	−

[a] Proline auxotrophs served as reporter strains for P5C or proline utilization. Strains were grown in rich medium then embedded in soft-agar overlaid on minimal growth medium lacking proline.

[b] Growth was assessed on minimal glucose medium containing arabinose (1.3 mM). Dap was added to the growth medium at a final concentration of 0.25 mM, where indicated.

[c] Producer strains were grown on NB agar. Single colonies were stabbed into agar overlays of the reporter strain.

[d] Cross-feeding was designated as growth (+) or no growth (−) based on turbidity of the reporter strain after 48 hours at 37˚C. Outgrowth of the producer strains (not shown) from the sites of inoculation is described in the text.