RidA proteins contribute to fitness of *S. enterica* and *E. coli* by reducing 2AA stress and moderating flux to isoleucine biosynthesis

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ABSTRACT Defining the physiological role of a gene product relies on interpreting phenotypes caused by the lack, or alteration, of the respective gene product. Mutations in critical genes often lead to easily recognized phenotypes that can include changes in cellular growth, metabolism, structure etc. However, mutations in many important genes may fail to generate an obvious defect unless additional perturbations are caused by medium or genetic background. The latter scenario is exemplified by RidA proteins. *In vitro* RidA proteins deaminate numerous imine/enamines, including those generated by serine/threonine dehydratase IlvA (EC:4.3.1.19) from serine or threonine – 2-aminoacrylate (2AA) and 2-aminocrotonate (2AC), respectively. Despite this demonstrable biochemical activity, a lack of RidA has little to no effect on growth of *E. coli* or *S. enterica* without the application of additional metabolic perturbation. A cellular role of RidA is to prevent accumulation of 2AA which, if allowed to persist, can irreversibly damage pyridoxal 5'-phosphate (PLP)-dependent enzymes, causing global metabolic stress. Because the phenotypes caused by a lack of RidA are dependent on the unique structure of each metabolic network, the link between RidA function and 2AA stress is difficult to demonstrate in some organisms. The current study used coculture experiments to exacerbate differences in growth caused by the lack of RidA in *S. enterica* and *E. coli*. Results described here solidify the established role of RidA in removing 2AA, while also presenting evidence for a role of RidA in enhancing flux towards isoleucine biosynthesis in *E. coli*. Overall, these data emphasize that metabolic networks can generate distinct responses to perturbation, even when the individual components are conserved.

INTRODUCTION

The robust metabolic network of a microbial cell requires the integration of individual components (i.e., enzymes and pathways) and multi-layered regulation of the resulting connections. In a general sense, the metabolic network is an amalgamation of interconnected biochemical pathways which are themselves composed of a diverse repertoire of enzymes. Cells modulate flux through these pathways by altering the expression or activity of appropriate enzymes which, in turn, dictate the rate and direction that relevant metabolites flow through the network. Precise control over cellular metabolism is allowed by the structural complexity of the system, emphasizing the notion that the individual parts have evolved to function within the context of the entire network. The precision allows microbial cells to survive in dynamic environments by restoring stability after a perturbation has been applied to the network.

Numerous components of metabolic networks in model bacteria are well characterized, although many enzymes and pathways remain poorly or only partially described. Historically, a first step in identifying the role of a gene is an analysis of phenotype(s) caused by the absence of the respective gene product. A detectable mutant phenotype demonstrates a physiological need for the gene and provides a tool to unravel the molecular function of the gene product. This approach loses its utility when a mutant has no detectable phenotype to guide functional studies. As a consequence, numerous genes remain uncharacterized, even in the best studied model systems. For example, the genome of *Escherichia coli* contains the coding capacity for over 4500 genes, an estimated 1200 of which have no associated phenotype or known function [1]. The characterization of these genes requires innovative experimental design and/or integration of data from genetic, biochemical and global analyses.
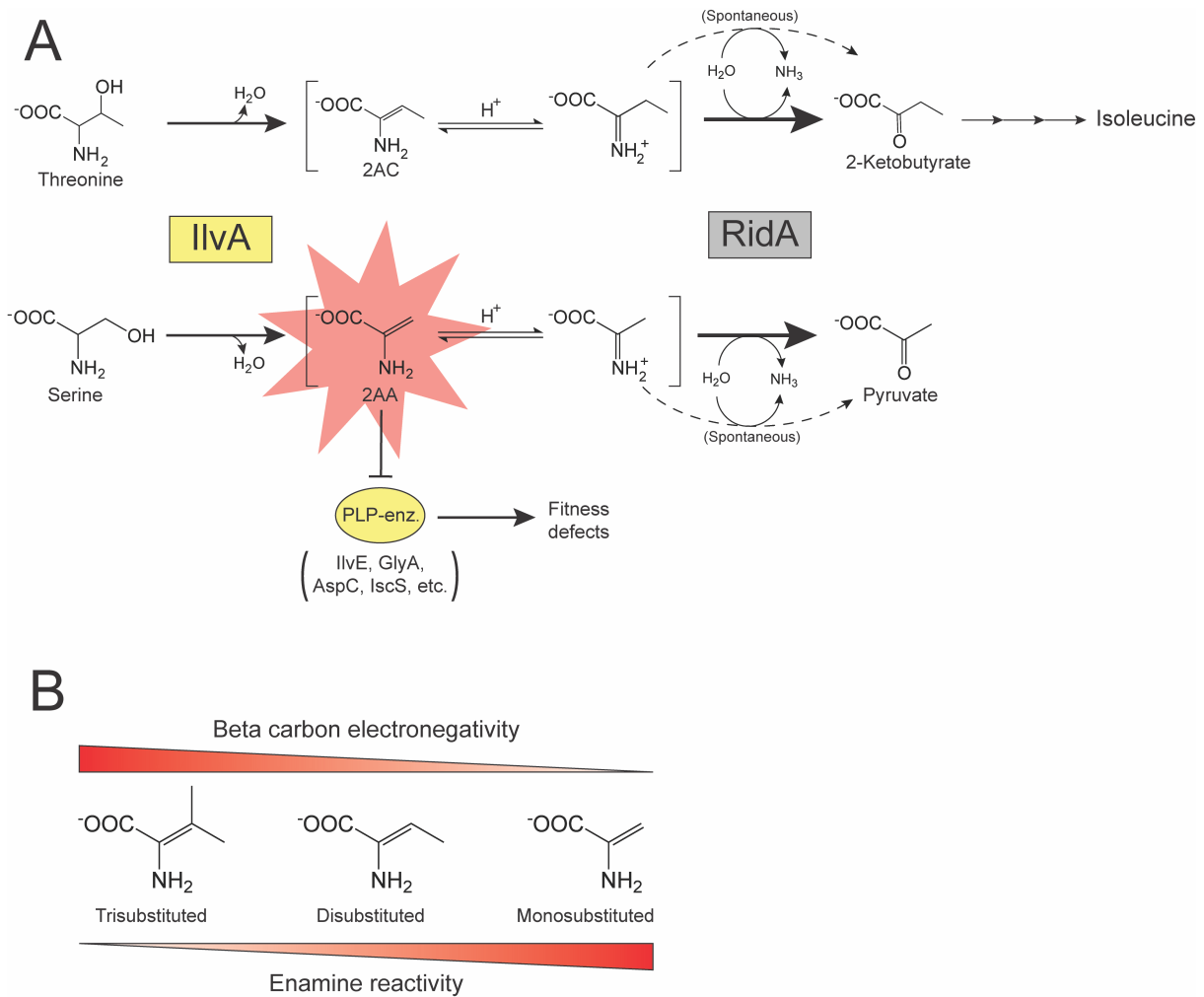
FiGURE 1 ● RidA paradigm and the effect of pi bond substitution on enamine reactivity. **(A)** RidA paradigm. IlvA (Serine/threonine dehydratase; EC:4.3.1.19) (yellow box) uses either threonine or serine as a substrate to generate 2AC or 2AA (red star), respectively. Enamine intermediates are spontaneously deaminated by water (dashed arrows) or by RidA (2-iminobutanoate/2-iminopropionate deaminase; EC:3.5.99.10) (grey box, bold arrows). 2AC is a disubstituted and benign enamine and has not been shown to damage cellular components in the absence of RidA. 2AA is a less substituted, more reactive enamine that can damage PLP-dependent enzymes (yellow oval), causing fitness defects if it persists in the cell. **(B)** Substitution of the pi bond between the α- and β-carbons increases stability of amino acid-derived enamines via increased electronegativity of the β-carbon.

Studies of the Rid family of proteins exemplify the difficulty in characterizing genes whose absence fails to cause a detectable phenotype in the laboratory. The Rid superfamily is divided into eight subfamilies [2], of which the RidA subfamily is the best characterized. In the past two decades, numerous experiments have probed the function of RidA across multiple model bacterial species. These studies identified RidA as an enamine/imine deaminase with at least two substrates of physiological significance, 2-aminoacrylate (2AA) and 2-aminocrotonate (2AC). The phenotypes of a *ridA* mutant that led to its functional characterization required further metabolic perturbations [3–5], or additional mutations [6–8]. The serine/threonine dehydratase IlvA (EC:4.3.1.19) generates 2AA and 2AC via the dehydration of serine or threonine, respectively [3, 9]. Past studies, primarily in *Salmonella enterica*, demonstrated that 2AA and 2AC accumulate in the absence of RidA. 2AA has been of particular interest due to its increased reactivity and toxicity compared to 2AC. 2AA has an unsubstituted pi bond between the α- and β-carbons,

which is less stable than the disubstituted pi bond of 2AC (**Figure 1B**). This feature makes the β-carbon of 2AA less electronegative, which will favor tautomerization and allow subsequent nucleophilic attack by this molecule [10]. The reactivity of 2AA facilitates attack on the pyridoxal 5′-phosphate (PLP) in the active site of some enzymes. Stress caused by 2AA accumulation results from decreased activity of several PLP-dependent enzymes that can cause growth defects in a *ridA* mutant (**Figure 1A**).

Over time, the RidA/2AA paradigm was described, target enzymes and metabolic consequences were identified, and suppressor mutations were isolated that minimized the impact of 2AA [11–18]. Features of this paradigm are present in several organisms, which led to the assumption that 2AA deaminase activity of RidA was the driver of its conservation and broad phylogenetic distribution [19, 20].

Recent observations have suggested that the deamination of 2AA may not be the sole cellular function of RidA. For instance,

in some organisms (*Bacillus subtilis*, *E. coli*) no evidence of naturally generated 2AA stress has been identified [21] (Buckner, unpublished). Further, RidA proteins deaminate diverse substrates *in vitro*, suggesting there may be additional physiologically relevant molecules acted on by RidA [20]. The latter possibility was prompted by the role of non-RidA members of the Rid family in deaminating imines that are not known to damage cellular components in a similar fashion as 2AA [22–24]. All reactions currently attributed to Rid proteins can also proceed non-enzymatically, if catalyzed by free water. However, in all described cases, the presence of a Rid protein increases the rate of product formation over that of the reaction catalyzed by water [2, 3, 19, 22–28]. In the case of RidA, the enhanced removal of 2AA ameliorates metabolic stress. An emerging pattern suggests that members of the Rid superfamily function to deaminate metabolic intermediates and increase flux through a particular metabolic pathway [2, 19, 22–24, 26]. For example, the Rid protein RutC is encoded in the pyrimidine utilization operon of *E. coli* and catalyzes the deamination of 3-aminoacrylate to malonate semialdehyde in a reaction previously designated as spontaneous [24, 29]. Additionally, members of the Rid2 subfamily in *Pseudomonas* species enhance flux through amino acid catabolic pathways, providing a competitive fitness advantage over strains lacking the Rid protein [22, 23].

The current study addressed the cellular role of RidA in *S. enterica* and *E. coli* in the context of a possible role in removing 2AC. 2AC is a physiologically relevant substrate of RidA at least in *S. enterica* and is not known to have toxic consequences [3, 8]. 2AC is an obligatory intermediate in the biosynthesis of isoleucine generated from threonine by IlvA. Results obtained from coculture experiments described herein confirm a role of RidA in preventing 2AA stress and suggest that RidA proteins may also have an organism-specific role in modulating biosynthetic flux to isoleucine.

RESULTS

S. enterica ridA mutants have a defect in competitive fitness

In minimal glucose medium, a *ridA* mutant has a slight, often immeasurable, growth defect in monoculture (**Figure 2**, **Figure 3**). Despite the lack of a significant growth phenotype, activity of branched chain aminotransferase (IlvE, EC:2.6.1.42) is decreased by ~40% in a *ridA* mutant under these conditions. This demonstrable biochemical defect indicates 2AA has accumulated [30–32]. Serine can be added to the medium to increase 2AA stress. The presence of serine prevents growth of a *ridA* mutant in *S. enterica* by partially inactivating serine hydroxymethyltransferase (GlyA, EC:2.1.2.1) and, to a lesser extent, cysteine desulfurase (IscS, EC:2.8.1.7) [5, 13, 18]. In competition experiments, *S. enterica ridA* mutants and wild type cells were co-inoculated into nutrient medium and minimal glucose medium. The competitive index (CI) for strains was calculated as the ratio of strains after growth in coculture normalized to the ratio present in the inoculum. The CI is a measure of *competitive* fitness that has typically been used to study the requirement for gene products when cells are grown in competition for a single limiting resource [22, 23]. In contrast,

experiments herein were designed to define competition under conditions where no single resource was limiting. In other words, CI values presented here define differences in general fitness (i.e., growth rate) of a strain that may not be apparent in monoculture experiments. No difference in fitness was detected between wild type and a strain lacking RidA grown in nutrient medium. In contrast, the *ridA* mutant had a significant fitness defect when the two strains were cocultured in minimal glucose medium. In minimal medium, the *ridA* mutant had a CI of 0.037 (ln(CI) = -3.3), which reflects a ~27-fold decrease in fitness compared to wild type (**Figure 2**). The significant fitness difference between wild type *S. enterica* and the *ridA* mutant amplified a growth difference that was minimal, or undetectable, in monoculture and underlined the sensitivity of this approach. Results from this competition assay aligned with the 2AA-dependent decrease in IlvE activity of a *ridA* mutant grown in minimal medium, validating that 2AA stress is present in *S. enterica* during growth in minimal medium [30, 31].

Isoleucine restores fitness of a *S. enterica ridA* mutant in coculture

Isoleucine in the growth medium restored full competitive fitness to the *ridA* mutant (**Figure 3**). This result was expected, as isoleucine is known to allosterically inhibit serine/threonine dehydratase IlvA and prevent the formation of 2AA [30, 31]. These data supported the idea that the fitness defect of a *ridA* mutant was due to accumulated 2AA.

A caveat to this simple interpretation is that RidA also deaminates the threonine-derived enamine product of IlvA, 2AC. 2AC is an obligatory intermediate in the biosynthesis of isoleucine that is eliminated when isoleucine inhibits IlvA. Thus, it was possible that RidA improved fitness of *S. enterica* by increasing biosynthetic flux to isoleucine. Distinguishing these two possibilities was complicated by the fact that isoleucine would eliminate the need for RidA in both scenarios. Coculture experiments to separate the possible contributions of RidA were based on the response of *S. enterica* to higher levels of 2AA stress generated when exogenous serine was added. The assumption was that the effect of 2AA stress generated by endogenous serine would be similar in consequence, if not magnitude, to that generated by addition of exogenous serine. Specifically, *S. enterica ridA* mutants grow well in the presence of 2AA stress if glycine and thiamine are provided. Together, these nutrients bypass the two key enzymes damaged by 2AA, GlyA and IscS and allow growth in the presence of 2AA stress [5, 13]. Notably, the presence of glycine and thiamine in the medium improved fitness of a *ridA* mutant in coculture with wild type, making them near equal (**Figure 3**). Importantly, a minor, but statistically significant difference in fitness remained. These data supported, but were not conclusive, that the competitive defect of a *ridA* mutant was caused by 2AA-dependent damage to the target enzymes GlyA and IscS. The slight fitness defect that remained left the possibility that either i) decreased flux to 2-ketobutyrate (from 2AC) in the isoleucine biosynthetic pathway contributes to the fitness defect of a *ridA* mutant, or ii) 2AA-dependent damage to PLP-dependent target enzymes beyond GlyA and IscS contribute to the fitness defect of a *ridA* mutant in *S. enterica*.
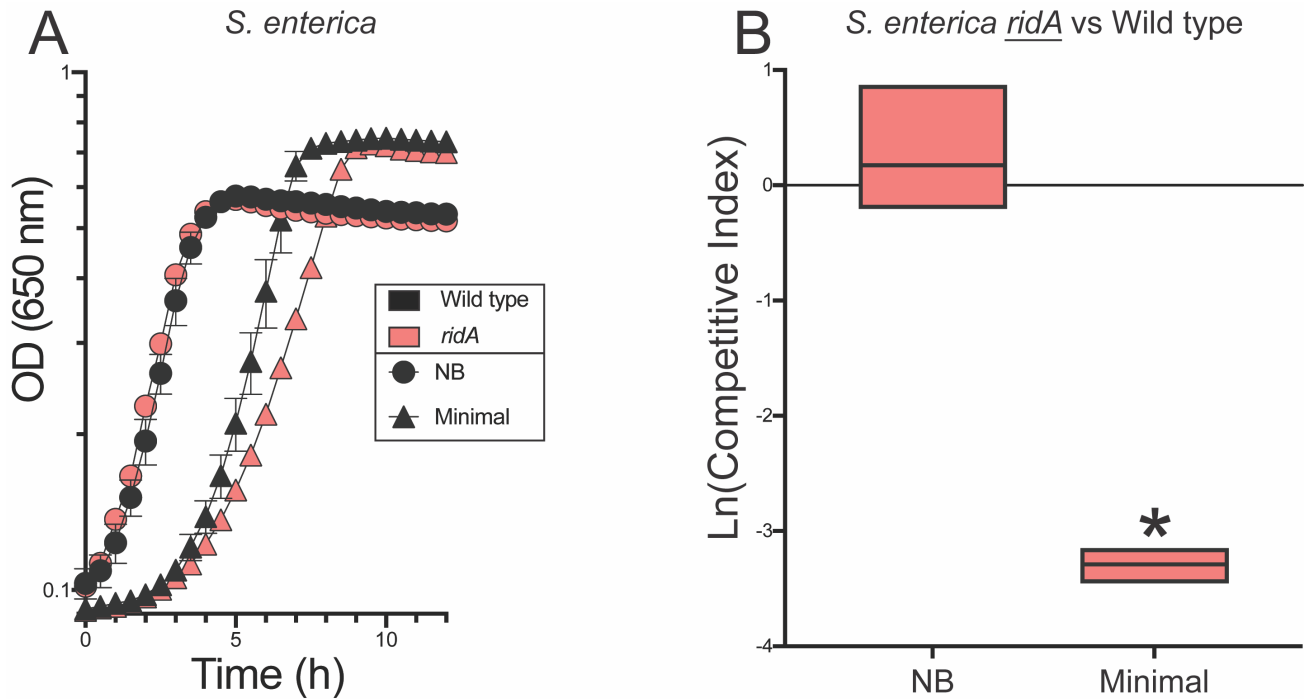
FIGURE 2 ● *S. enterica ridA* mutants have a fitness defect in minimal medium. (A) Growth of *S. enterica* wild type (black) and a *ridA* mutant (red) in nutrient broth (NB, circles) and minimal glucose (11 mM) medium (triangles). (B) Competitive fitness of a *S. enterica ridA* mutant (*ridA3*::MudJ) grown in competition with wild type *S. enterica* in nutrient or minimal medium. The underlined genotype indicates the strain for which the competitive indices are shown in the corresponding graph. An asterisk (∗) indicates a significant difference (p ≤ 0.05 as determined by Sidak's multiple comparisons test) in CI values compared to control experiments in which the *ridA* mutant was grown in competition with a second *ridA* mutant (*ridA4::Cm*) in the relevant medium. Data shown are the mean and standard deviation of three independent biological replicates.
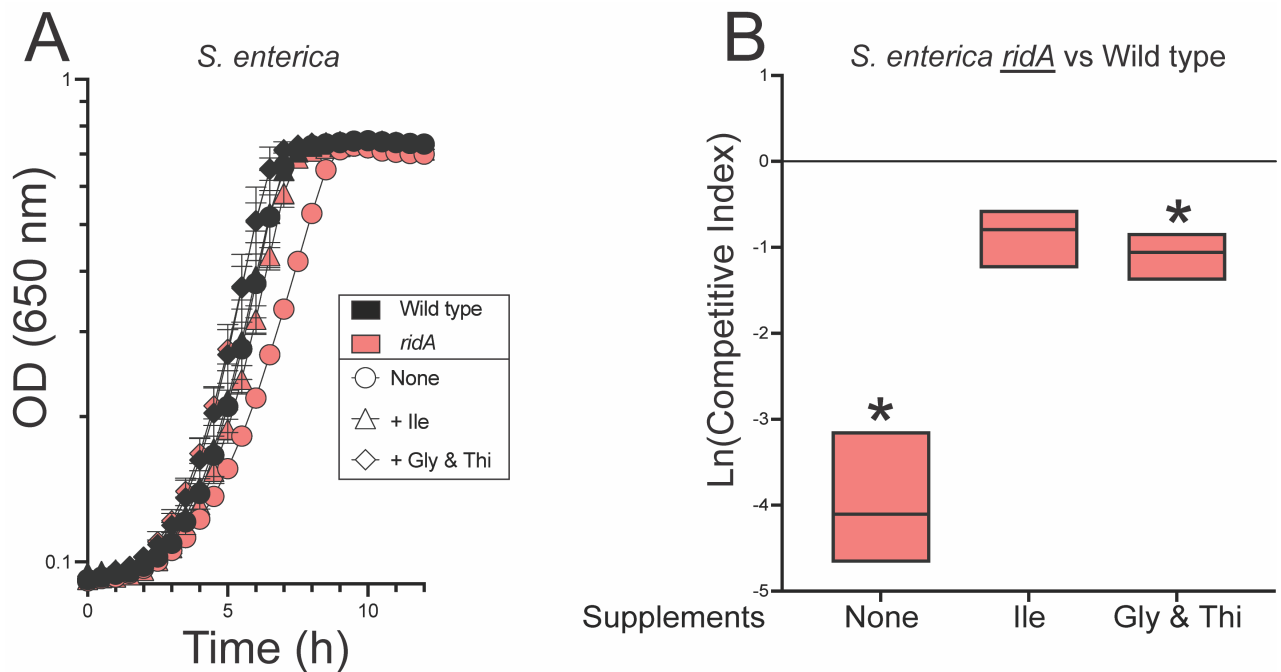

FIGURE 3 ● Glycine and thiamine alleviate fitness defects of *S. enterica ridA* mutants. (A) Growth of *S. enterica* wild type (black) and a *ridA* mutant (red) in minimal glucose (11 mM) medium supplement with nothing (circles), 1 mM L-isoleucine (triangles) or 1 mM glycine and 100 nM thiamine (diamonds). (B) Competitive fitness of a *S. enterica ridA* mutant (*ridA3*::MudJ) grown in competition with wild type *S. enterica* in minimal glucose medium with indicated supplements. An asterisk (∗) indicates a significant difference (p ≤ 0.05 as determined by Sidak's multiple comparisons test) in CI values compared to control experiments in which the *ridA* mutant was grown in competition with a second *ridA* mutant (*ridA4::Cm*) in the indicated medium. Data shown are the mean and standard deviation of three independent biological replicates.
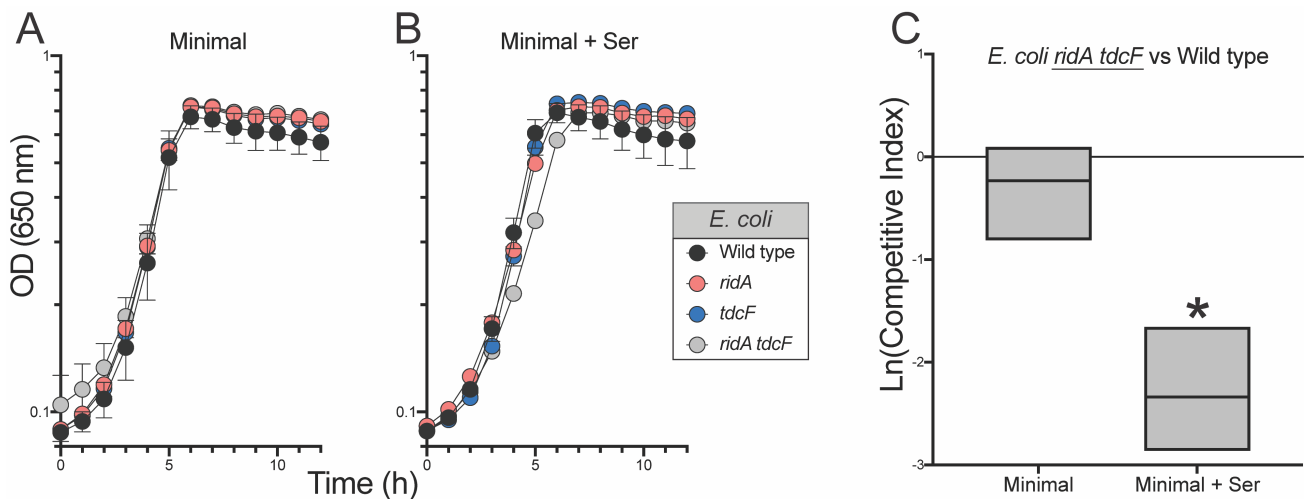
FiGURE 4 ● RidA proteins are required for full fitness of *E. coli.* (A and B) Shown are growth profiles of wild type *E. coli* (black), a *ridA* mutant (red, *ridA790::Km*), *tdcF* mutant (blue, *tdcF12::Km*) and a *ridA tdcF* double mutant (grey, Δ*ridA890tdcF13::Cm*). Strains were grown in (A) minimal glucose (11 mM) medium with no supplementation or (B) supplemented with 5 mM L-serine. (C) Shown is competitive fitness of an *E. coli ridA tdcF* double mutant (Δ*ridA890 tdcF13::Cm*) grown in coculture with wild type *E. coli* in minimal glucose (11 mM) medium with or without L-serine (5 mM). Data shown are mean and standard deviation of three independent biological replicates. The underlined genotype indicates the strain for which the CIs are shown in the corresponding graph. An asterisk (∗) indicates a significant difference (p ≤ 0.05 as determined by Sidak's multiple comparisons test) in CI values compared to control experiments in which Δ*ridA890 tdcF13::Cm* was competed with an different strain lacking *ridA* and *tdcF* (Δ*ridA890* Δ*tdcF14*) in the indicated medium.

RidA proteins are required for full fitness of *E. coli*

Past studies have identified multiple differences between the metabolic networks of *S. enterica* and *E. coli* [21, 33, 34]. *E. coli* encodes two RidA proteins, RidA and TdcF, which have been assessed for a role in 2AA stress [21]. The *ridA* gene is monocistronic, while *tdcF* is in the *tdc* operon that encodes enzymes involved in a putative threonine catabolic pathway [35, 36] and are thought to be expressed only under anaerobic conditions [37, 38]. Evidence of 2AA stress was observed in *E. coli* only with significant manipulation of the genome and growth medium. Loss of TdcF and/or RidA had no observable effect on growth nor activity of known 2AA target enzymes, unless 2AA stress was artificially intensified by expression of a feedback-insensitive variant of IlvA (encoded by the *ilvA219* allele) in the presence of exogenous serine [21].

Unlike *S. enterica*, the absence of RidA proteins in *E. coli* (RidA and TdcF) had no effect on the growth, or competitive fitness of strains grown in minimal medium. In medium supplemented with 5 mM serine, the *ridA tdcF* double mutant strain had a small, but statistically significant, difference in growth rate (**Figure 4**) reminiscent of *S. enterica ridA* mutants grown in minimal glucose medium. Despite the minimal growth difference in monoculture, the *ridA tdcF* mutant was notably less fit than wild type in minimal medium supplemented with serine (**Figure 4**). Unlike the situation in *S. enterica*, there was no significant defect in IlvE activity of the *E. coli tdcB ridA* mutant when grown under these conditions that produced a clear fitness defect (5 mM serine) (**Table 1**).

Additional competition experiments showed that RidA was the homolog critical for full fitness of *E. coli* grown in minimal medium with 5 mM exogenous serine (**Figure 5**). There was no difference between the fitness of the *ridA tdcF* double mutant and a *ridA* mutant. In contrast, the *ridA tdcF* double mutant was less fit than a *tdcF* mutant when grown in coculture. Isoleucine

restored full fitness to the *E. coli* strains, eliminating the effect of the *ridA* mutation as found in *S. enterica*. These data were consistent with a scenario that 2AA stress negatively impacted fitness of *E. coli*.

2AA stress is induced in *E. coli* with increased serine concentration

The above results could be explained in a scenario where 5 mM serine generated enough 2AA to compromise fitness, but not enough to cause detectable damage to target enzyme IlvE. If this was the case, increased concentrations of serine might increase 2AA production to a level that could generate a growth defect in monoculture. Consistently, both the *ridA* and *ridA tdcF* mutants had a growth defect with 20 mM serine, while the growth of wild type was not affected (**Figure 6**). The serine-induced growth defects of the *ridA* and *ridA tdcF* mutants were eliminated with the addition of isoleucine or glycine, consistent with the pattern in *S. enterica* with 2AA-dependent growth defects. As expected based on the growth defects in monoculture, *E. coli ridA* and *ridA tdcF* mutants were outcompeted by wild type in minimal medium containing 20 mM serine, and the *ridA tdcF* mutant having a more severe defect than the *ridA* single mutant (**Figure 8**). The addition of isoleucine restored full fitness to each stain in the presence of 20 mM serine. The observation that TdcF had an impact on fitness in the presence of elevated serine concentration was not entirely unexpected. *E. coli* strains with synthetically increased 2AA levels exhibited a growth defect only when both TdcF and RidA were absent, consistent with their redundant activities [21]. In total, the results with *E. coli* suggested that either i) 2AA levels are generally lower in *E. coli* than *S. enterica* when strains lack RidA proteins, and/or ii) 2AA is less detrimental to the metabolic network of *E. coli* than it is to that of *S. enterica*. Neither

TABLE 1 ● 2AA stress is not detectable in *E. coli* strains lacking RidA proteins.

Strain	Relevant Genotype	Branched Chain Amniotransferase Activity*		
		Minimal	Minimal + Ser	Minimal + Ser + Ile
DM14520	Wild type	130 ± 6	132 ± 4	243 ± 6
DM14597	*ridA*	140 ± 20	110 ± 4	234 ± 23
DM14699	*tdcF*	163 ± 15	163 ± 5	265 ± 22
DM14682	*ridA tdcF*	131 ± 9	134 ± 7	220 ± 4

*Activity of Branched Chain Aminotransferase (IlvE) is reported as nmol 2KMV/mg protein.

E. coli strains were grown in minimal glucose medium with the addition of serine (5 mM) with or without isoleucine (1 mM) as indicated. The activity of IlvE was measured as described in Experimental Procedures. Data shown are the mean and standard error of two biological replicates assayed in three technical replicates. There was no significant difference in IlvE activity between wild-type and mutant strains grown in the same medium, or between growth in minimal with and without serine addition. IlvE activity was significantly higher when grown in the presence of serine and isoleucine than the same strain in the other media tested.
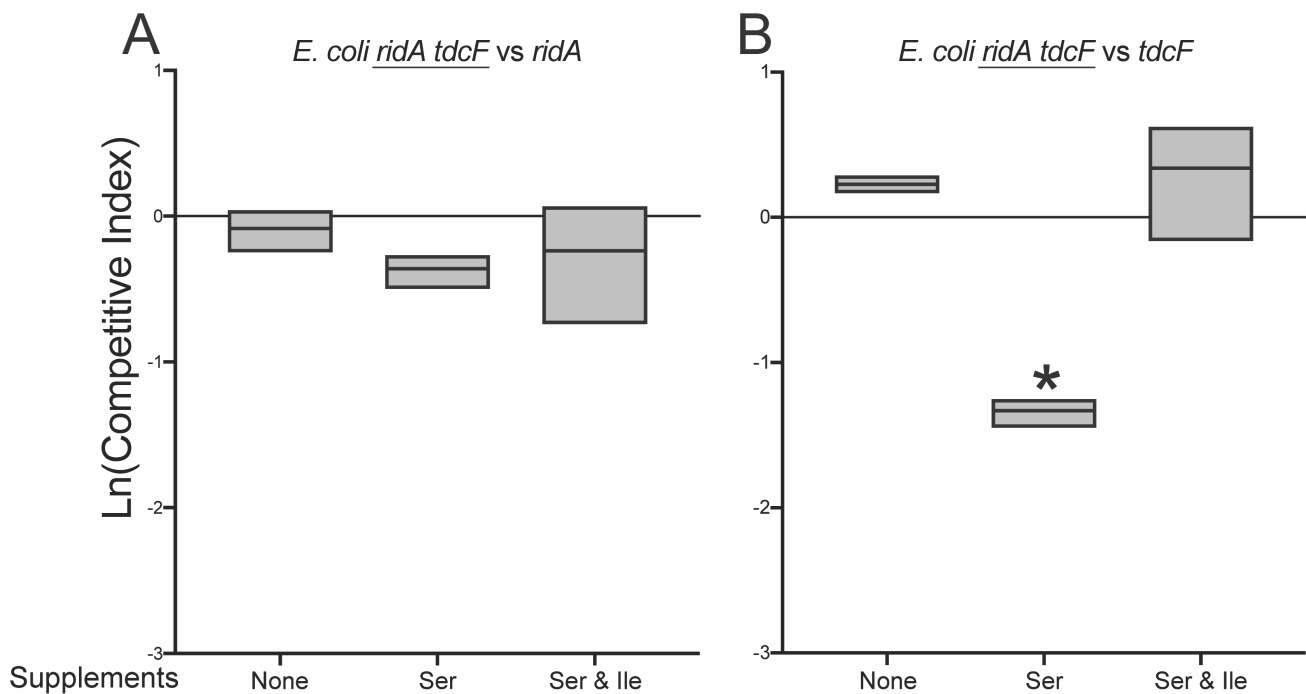

FiGURE 5 ● RidA contributes to the competitive fitness of *E. coli*. Shown are competitive fitness values for an *E. coli ridA tdcF* double mutant ($\Delta ridA890$ *tdcF13::Cm*) grown in coculture with (A) an *E. coli ridA* mutant (*ridA790::Km*) or (B) a *tdcF* mutant (*tdcF12::Km*) in minimal glucose (11 mM) medium with the indicated supplements. L-serine was provided at a final concentration of 5 mM and L-isoleucine each provided at 1 mM. Data shown are mean and standard deviation of three independent biological replicates. The underlined genotype indicates the strain for which the CIs are shown in the corresponding graph. An asterisk (∗) indicates a significant difference (p ≤ 0.05 as determined by Sidak's multiple comparisons test) in CI values compared to control experiments in which $\Delta ridA890tdcF13::Cm$ was competed with an isogenic strain also lacking *ridA* and *tdcF* ($\Delta ridA890 \Delta tdcF14$) under each condition.

of these directly addressed the disconnect between the lack of detectable 2AA damage (i.e., IlvE activity) and competitive fitness defect of an *E. coli ridA* mutant in minimal medium with 5 mM serine.

RidA has a role beyond 2AA stress control in *E. coli*

The data gathered for *ridA* mutants of *E. coli* appeared consistent with a model in which, i) the fitness defect on 5 mM serine is due to 2AA stress, despite the stress being too low to impair IlvE activity, ii) high levels of exogenous serine (20 mM) generate enough 2AA to cause a growth defect, and iii) glycine and isoleucine restore growth via the mechanism(s) characterized in *S. enterica ridA* mutants. Based on this scenario,

glycine was expected to suppress the competitive fitness defect of the *ridA tdcF* mutant. Unexpectedly, glycine failed to improve fitness and the *ridA tdcF* mutant remained less fit than wild type in medium with serine (5 mM) and glycine (Figure 7). Furthermore, the addition of glycine did not restore competitive fitness defects of *ridA* or *ridA tdcF* mutants in the presence of 20 mM serine, despite its ability to restore growth in monoculture (Figure 8). A possible explanation for these results is that, in *E. coli* RidA has a role in optimizing flux through isoleucine biosynthesis. In this scenario, isoleucine contributes to restoring fitness by satisfying a need for isoleucine created by sluggish *de novo* synthesis, in addition to its role in decreasing 2AA generation. The finding that glycine eliminates the 2AA-generated growth defect in monoculture, while failing to
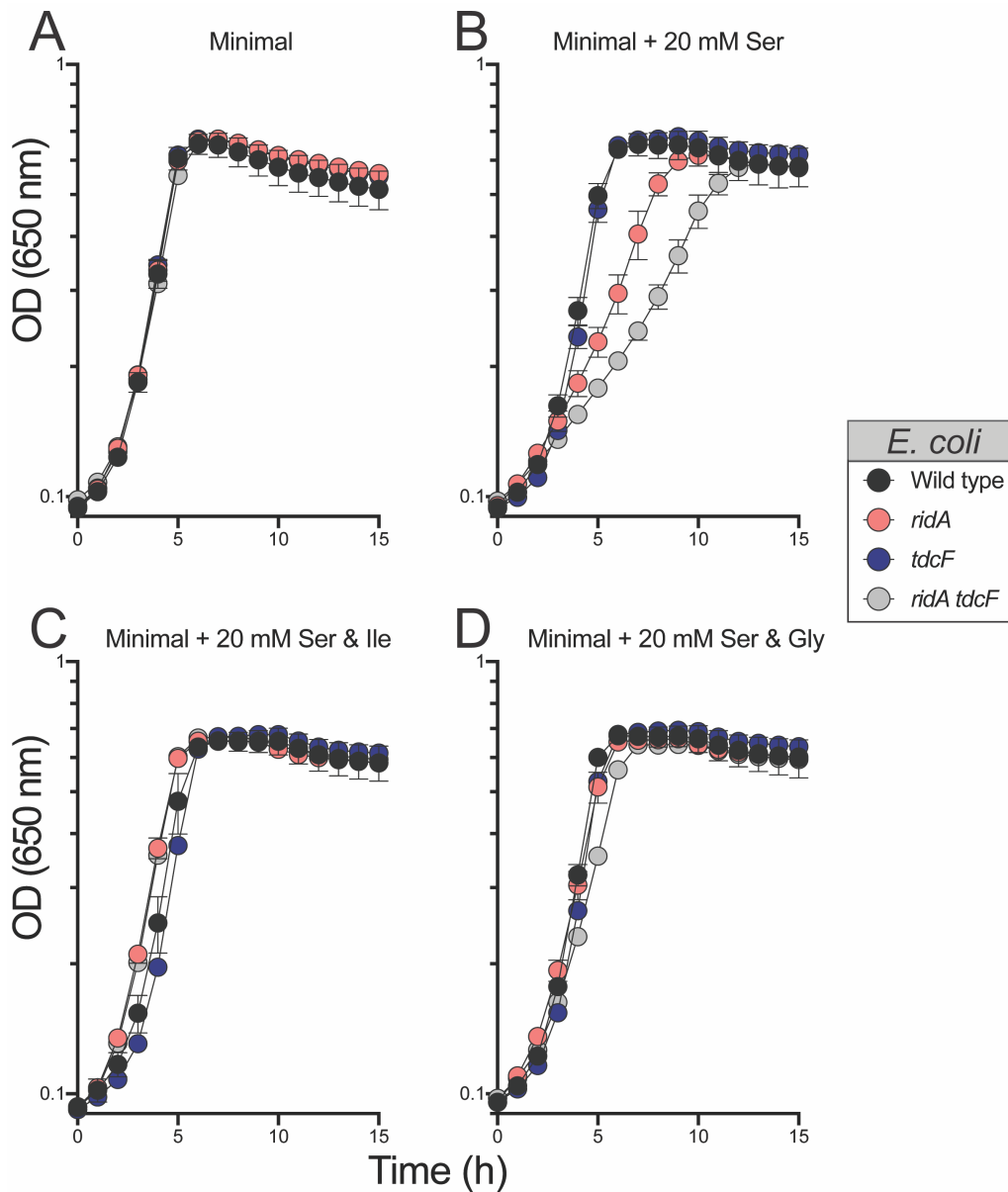
FiGURE 6 ● **Glycine does not restore competitive fitness to *E. coli ridA tdcF* double mutants under 2AA stress.** Shown is competitive fitness of an *E. coli ridA tdcF* double mutant (Δ*ridA890 tdcF13::Cm*) grown in coculture with wild type *E. coli* in minimal glucose (11 mM) medium with the indicated supplements – L-serine (5 mM), glycine (1 mM), L-isoleucine (1 mM). Data shown are mean and standard deviation of three independent biological replicates. The underlined genotype indicates the strain for which the competitive indices are shown in the corresponding graph. An asterisk (⋆) indicates a significant difference (p ≤ 0.05 as determined by Sidak's multiple comparisons test) in CI values compared to control experiments in which Δ*ridA890tdcF13::Cm* was competed with an isogenic strain lacking *ridA* and *tdcF* (Δ*ridA890 Δ tdcF14*) in each medium.

restore the fitness defect of the *ridA tdcF* mutant, supports the conclusion that decreased fitness of *E. coli* on minimal medium with serine is not solely due to 2AA stress.

The kinetics of $_{EC}$IlvA model 2AA and 2AC production

The above data suggested that fitness defects of *E. coli ridA* and *ridA tdcF* mutants in coculture were not solely due to 2AA-mediated damage to PLP-dependent enzymes. The deaminase activities of other members of the Rid protein family improve flux through their respective pathway(s) and thus increase fitness. Based on this precedent, it was considered possible that fitness defects of *E. coli* mutant strains in coculture were due

to impaired flux to isoleucine in the absence of RidA. For this to be the case, the data indicated flux was reduced only when exogenous serine was present. In principle, impaired flux could be due to i) decreased 2AC formation by IlvA, or ii) decreased deamination of 2AC to 2KB in the absence of RidA. Only the former possibility appeared to be amenable to an effect by serine, since competition between serine and threonine for IlvA could potentially alter the 2AC:2AA ratio. In *S. enterica*, kinetic models indicated that the production of 2AC from threonine was not affected by the level of serine present *in vivo* [39]. The catalytic parameters (K_M and k_{cat}) of the *E. coli* protein ($_{EC}$IlvA)
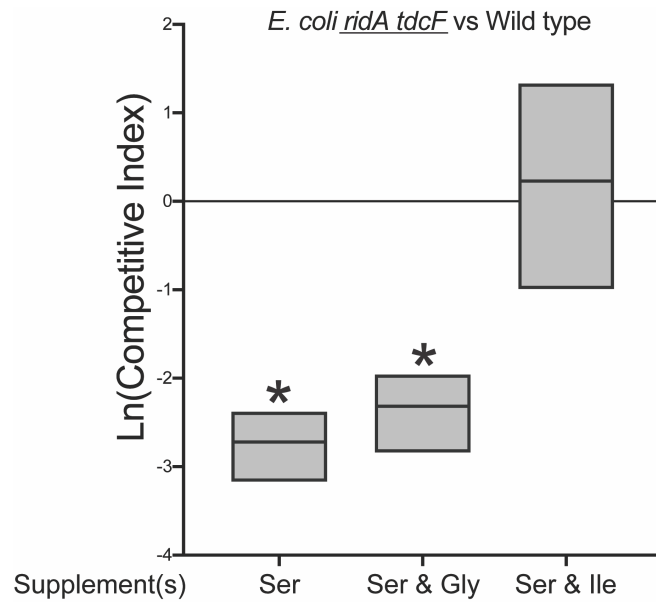
FIGURE 7 ● Glycine does not restore competitive fitness to *E. coli ridA tdcF* double mutants under 2AA stress. Shown is competitive fitness of an *E. coli ridA tdcF* double mutant ($\Delta ridA890$ *tdcF13::Cm*) grown in coculture with wild type *E. coli* in minimal glucose (11 mM) medium with the indicated supplements – L-serine (5 mM), glycine (1 mM), L-isoleucine (1 mM). Data shown are mean and standard deviation of three independent biological replicates. The underlined genotype indicates the strain for which the competitive indices are shown in the corresponding graph. An asterisk (*) indicates a significant difference ($p \leq 0.05$ as determined by Sidak's multiple comparisons test) in CI values compared to control experiments in which $\Delta ridA890$ *tdcF13::Cm* was competed with an isogenic strain lacking *ridA* and *tdcF* ($\Delta ridA890 \Delta tdcF14$) in each medium.
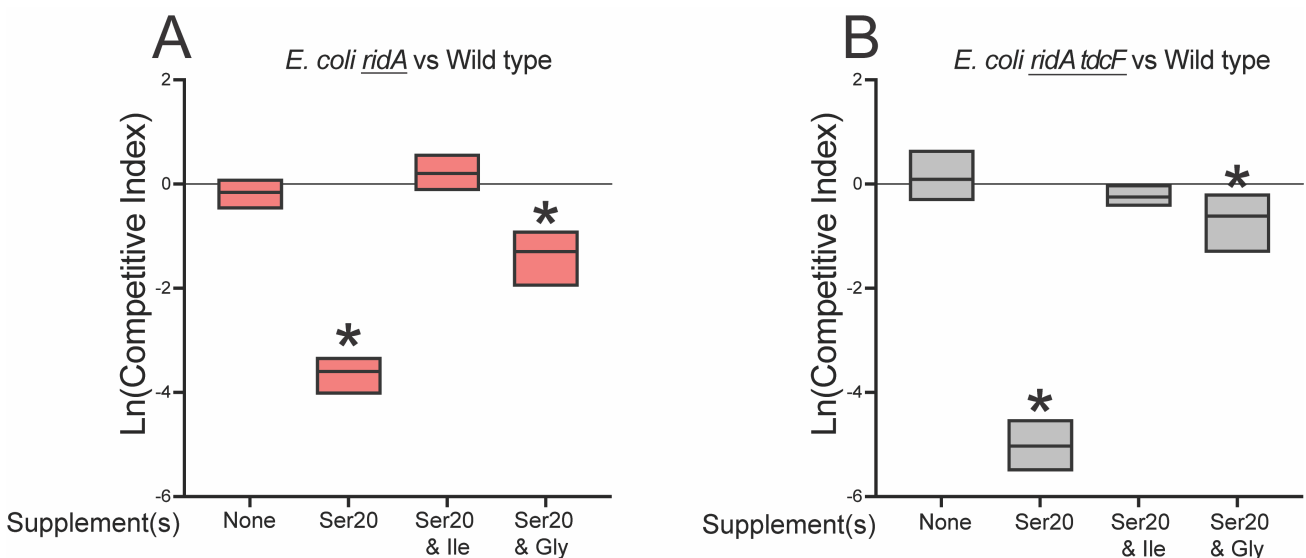

FIGURE 8 ● Glycine does not restore competitive fitness to *ridA* or *ridA tdcF* mutants in the presence of elevated serine concentrations. Shown are competitive indices of (A) an *E. coli ridA* mutant (*ridA790::kan*) and (B) a *ridA tdcF* double mutant ($\Delta ridA890$ *tdcF13::Cm*) grown in coculture with wild type *E. coli* in minimal glucose (11 mM) medium with the indicated supplements – L-serine (20 mM), glycine (1 mM), L-isoleucine (1 mM). Data shown are mean and standard deviation of three independent biological replicates. The underlined genotype indicates the strain for which the CIs are shown in the corresponding graph. An asterisk (*) indicates a significant difference ($p \leq 0.05$ as determined by Sidak's multiple comparisons test) in CI values compared to control experiments in which each strain was competed with an isogenic strain lacking the relevant RidA homolog(s) in each medium.

were determined with serine and threonine as substrates (Figure S1). Using these parameters of $_{EC}$IlvA to model endogenous 2AC and 2AA production in *E. coli* indicated that 2AC production was not impacted by the increased production of 2AA (**Table 2**). The ratios of 2AC:2AA predicted by modeling were consistent between *S. enterica* and *E. coli*. In total, these data show that while 2AC genertion is not predicted to differ, the amount of 2AA relative to 2AC increases dramatically in *E. coli* strains grown with serine. In total the data are consistent with a scenario in which 2AA stress is not the sole cause of fitness defects in strains lacking RidA homologs. Fitness defects may be exacerbated by impaired flux through isoleucine biosynthesis that results from lower 2AC deamination in the absence of RidA activity, the direct effect of serine on this flux remains unclear.

DISCUSSION

In *S. enterica*, *ridA* is one of many genes that do not generate an observable, or easily quantifiable, phenotype unless perturbations are applied to the system. In this study, we used cocultures to exacerbate minor growth defects and generate a quantifiable fitness phenotype for *ridA* mutants. Unlike canonical competition analyses that define fitness in terms competing for a single limiting resource, coculture experiments in this study defined fitness of a mutant compared to wild type for overall growth (requiring synthesis of all cellular components) in a defined minimal medium. This approach provided a sensitive assay for the detrimental effects of 2AA that were not detected during growth in monoculture.

In *S. enterica*, the requirement for RidA is revealed by adding serine to the growth medium. The presence of serine increases IlvA-mediated formation of the reactive enamine, 2AA. In the absence of added serine, 2AA stress in a *ridA* mutant is evidenced by decreased IlvE activity. Data herein allow the conclusion that 2AA stress decreases fitness of a *S. enterica* *ridA* mutant in minimal medium, despite the full growth of the mutants in monoculture. The restoration of fitness by isoleucine or glycine and thiamine supported this conclusion but did not eliminate the possibility of additional metabolic complexity.

Results with *E. coli* suggested a more nuanced situation than the 2AA stress paradigm defined with *S. enterica*. First, *E. coli* strains lacking RidA proteins had a significant fitness defect under conditions (i.e., minimal medium with 5 mM serine) where there was no quantifiable evidence of 2AA stress – i.e., lowered IlvE activity. Secondly, while the fitness defect was eliminated by the presence of isoleucine, glycine had little effect, further distinguishing the behavior of *E. coli* from *S. enterica*. Importantly, glycine restored growth of the *ridA tdcF* mutants of *E. coli* when 2AA levels were increased with elevated (20 mM) serine. Thus, if the competitive defect of the *ridA tdcF* mutant was caused by 2AA stress alone, glycine might be expected to completely restore fitness in coculture, which it did not. In total, the data suggest that the role of RidA proteins, at least in *E. coli*, extends beyond preventing accumulation of 2AA. Specifically, results were consistent with a scenario in which RidA has an additional role of increasing flux through *de novo* synthesis of isoleucine.

The experiments with *E. coli* suggested an expanded role for RidA, while raising additional questions about the structure of the metabolic network. Results of competition experiments suggested that RidA was needed to optimize flux to isoleucine in the presence of serine, but not in minimal medium. The kinetic parameters of $_{EC}$IlvA, much like that of $_{SE}$IlvA [39], suggest 5 mM serine increases the amount of 2AA without affecting the amount of 2AC generated in the cell. Therefore, the need for RidA to contribute to biosynthetic flux only in the presence of serine seems to be due to the ratio of 2AA/2AC, though the precise mechanism remains unclear. The lack of a quantifiable phenotype indicates the increase in 2AA mediated by 5 mM serine does not cause detectable damage to target enzymes in *E. coli*. It is possible that a higher ratio of 2AA:2AC amplifies a need for RidA to generate 2-ketobutyrate at a rate that generates isoleucine efficiently. Such efficiency appears to be important only when in coculture with a strain fully proficient at isoleucine synthesis, due to the presence of RidA. Consider that 2AC has three fates in the *E. coli* cell, two of which lead to isoleucine, and one contributes to the synthesis of thiamine via TrpD forming phosphoribosylamine (PRA) from 2AC and phosphoribosylpyrophosphate (PRPP) [8] (**Figure 9**). The metabolic node encompassing 2AC and PRA is structured differently in *S. enterica* and *E. coli*, consistent with a difference in the role of RidA in the two organisms [6, 9, 33, 40].

It is worth noting that a previous study increased the accumulation of 2AA in *E. coli* by utilizing both serine and a variant of IlvA that was insensitive to allosteric inhibition by isoleucine [21]. Under these conditions, 2AA stress that compromised growth in *E. coli* in monoculture was generated, and glycine failed to restore growth. This result apprears inconsistent with the scenario proposed here. However, IlvA is expected to be critical in generating appropriate ratios of reactive intermediates (i.e., 2AA and 2AC) which, in turn, can influence flux through numerous metabolic pathways. Because of this connection, eliminating allosteric regulation of IlvA will have consequences beyond the specific accumulation of 2AA, offering an explanation for the differences found in the two studies.

This study, like others that have dissected metabolic integration, emphasizes the significance of metabolic network structure in defining the response of each organism to perturbation. The response of an organism to metabolic network perturbations must be defined on a case-by-case basis. This realization highlights the fact that metabolism is a complex system that has been finely tuned by evolution, rather than a network assembled fortuitously from components that evolved for individual efficacy. Specific to this work, *E. coli* experiences less 2AA accumulation and/or the metabolic network is more resistant to the resulting damage, while the synthesis of isoleucine in this organism can be a limiting factor under some growth conditions. In contrast, *S. enterica* is sensitive to 2AA accumulation, yet the biosynthesis of isoleucine appears to be sufficient for fitness even in the absence of RidA. These results reflect differential responses of two metabolic networks to perturbation, in spite of the similarity of individual components. Defining the consequences of perturbation in multiple systems (i.e., organisms) will continue to expand our knowledge of the breadth of strategies that are germane when modeling metabolism.

TABLE 2 ● Modeled rates of product formation by $_{EC}$IlvA.

[Serine] (μM)	[Threonine] (μM)	2AA formation (μM/s)	2AC formation (μM/s)
68	180	6	84
5000	180	398	81
20000	180	1410	83

Modeled rates of endogenous product formation by $_{EC}$IlvA using varied concentrations of serine and threonine. Data were calculated with an IlvA active site concentration of 10 mM, along with relevant k_{cat} and K_M values for each substrate (Figure S1) using the formula, and the substrate concentrations described in the Materials and Methods section. Higher serine concentrations were used assuming that the exogeneous concentration was equaled endogenously.
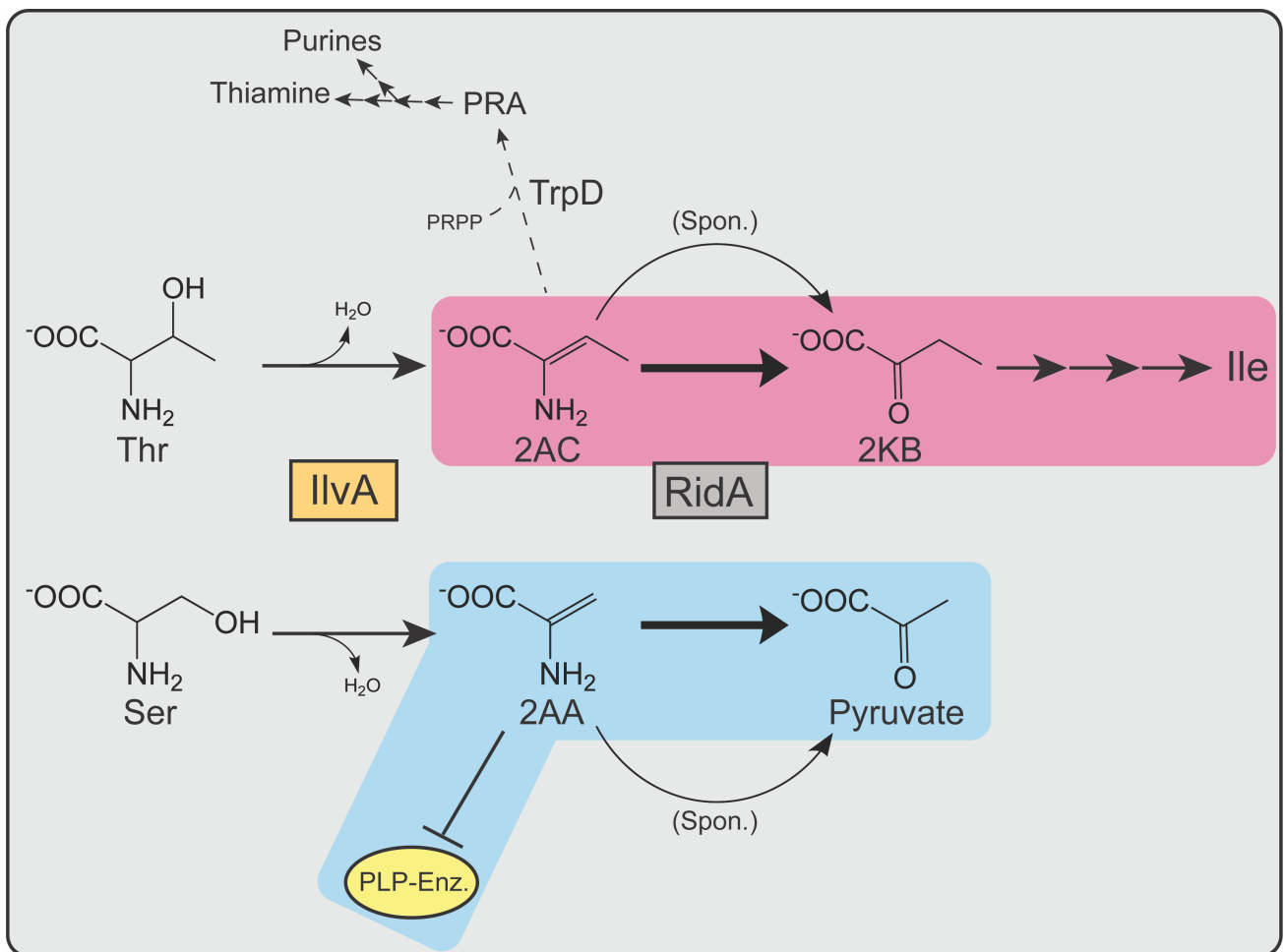

FiGURE 9 ● **RidA proteins contribute to fitness in *S. enterica* and *E. coli* by moderating flux through different metabolic nodes.** Shown are the pathways relevant to RidA activity in *S. enterica* and *E. coli*. IlvA (orange box) dehydrates serine or threonine, generating 2AA or 2AC, respectively. 2AA and 2AC are spontaneously deaminated by water (curved arrows) or, more rapidly, by RidA proteins (grey box, bold arrows) to the corresponding ketoacid product – pyruvate or 2KB. 2KB serves as a substrate for Ile synthesis. 2AA can irreversibly damage PLP-dependent enzymes (yellow oval) in the absence of RidA. Metabolic nodes in which RidA activity primarily contributes to fitness of each organism are highlighted in blue (*S. enterica*) or magenta (*E. coli*). 2AC, along with PRPP, is also used as a substrate by TrpD (dashed arrow) to generate PRA, a precursor to purines and thiamine.

MATERIALS AND METHODS

Strains, media, and chemicals

Strains used in this study are derivatives of *Salmonella enterica* serovar Typhimurium LT2 or *Escherichia coli* K-12 and are listed in Table 3. All strains were grown at 37°C unless otherwise stated. *S. enterica* strains were routinely grown in Nutrient Broth (NB; Difco; 8 g/liter, 5 g/liter NaCl). *E. coli* strains were grown in Lysogeny Broth (LB; 10 g/liter tryptone, 5 g/liter NaCl, 5 g/liter yeast extract) as a rich medium. No-carbon E (NCE) medium with 11 mM glucose, supplemented with 0.1 mM $MgSO_4$ and trace elements was used as a minimal medium [41]. Other supplements were added as noted to the following concentrations: L-isoleucine (1 mM), L-glycine (1 mM), thiamine (100 nM), L-aspartate (1 mM), and L-serine (5 mM). Antibiotics were used as indicated: kanamycin (Km; 50 μg/ml for rich media or 12.5 μg/ml for minimal media) and chloramphenicol (Cm; 20 μg/ml for rich media or 5 μg/ml for minimal media). Chemicals were purchased from MilliporeSigma (St. Louis, MO).

Growth analyses

Cultures were started from a single colony and grown overnight in rich medium with shaking at 37°C (2 ml). Cells were pelleted and resuspended in an equal volume of sterile saline. Cell suspensions (5 μl) were used to inoculate the indicated medium (195 μl). Growth was monitored in a 96-well plate as the change in optical density at 650 nm (OD_{650}) over time using a BioTek ELx808 plate reader (BioTek Instruments, Winooski, VT) with a slow shaking speed (210 rpm). Data were plotted using Graphpad Prism version 8.4. Growth experiments were performed with three independent biological replicates unless otherwise stated.

Competition assays

Competition assays were performed generally as described [22, 44, 45]. Briefly, overnight cultures of each strain were grown in rich medium (2 ml) at 37°C with shaking. Cells were pelleted and resuspended in 2 ml sterile saline and optical densities at 650 nm (OD_{650}) were normalized to 0.1. 5 ml of the indicated medium was inoculated with 50 μl of cell suspension from each of the two competing strains. Control experiments were performed for each condition in which strains of the same relevant genotype, with different antibiotic resistance cassettes, were competed against each other. These controls accounted for differences in competitive fitness caused by the different antibiotic resistance cassettes in each strain. Colony forming units (CFUs) of cocultures were determined by spreading serial dilutions at T=0 (i.e., before incubation of the cocultures) onto media to differentiate between each respective strain. For *S. enterica*, media were as follows: (i) minimal medium supplemented with L-serine, selective for wild type (DM9404); (ii) minimal medium supplemented with chloramphenicol, selective for a chloramphenicol-resistant ridA mutant (DM17845), and (iii) minimal medium supplemented with kanamycin, selective for a kanamycin-resistant ridA mutant (DM3480). For *E. coli*, media were as follows: (i) LB supplemented with chloramphenicol, selective for chloramphenicol-resistant *ridA* and *ridA tdcF* mutants (DM14820, DM14682), and (ii) LB supplemented with kanamycin, selective for kanamycin-resistant *ridA* and *tdcF*

mutants (DM14597, DM14669). Some *E. coli* strains (DM14520, DM15051) did not contain antibiotic resistance cassettes and could not be isolated from cocultures by spreading onto selective media. CFUs of these strains were determined by counting CFUs on LB media and subtracting the CFUs of the competing strain, which could be selected for with antibiotics. Cocultures were grown to stationary phase at 37°C with shaking. CFUs were enumerated for cultures after co-incubation as described above. The competitive index (CI) was defined and calculated as follows:

$$CI = \frac{(\frac{CFU\ mutant}{CFU\ wild\ type})_{initial}}{(\frac{CFU\ mutant}{CFU\ wild\ type})_{final}}$$

Each competition assay was performed at least three times with similar results. GraphPad Prism version 8.4 was used for data plotting and statistical analysis.

Protein purification

E. coli K12 *ilvA* was cloned into BspQI sites of pTEV18 as described previously. The resulting plasmid, pTEV18-*ilvA* (K12), was electroporated into BL21-AI. The resulting strain (DM18490) was grown overnight in 40 ml of Terrific Broth (TB; 12 g/liter tryptone, 24 g/liter yeast extract, 0.4% glycerol (v/v)) supplemented with 150 mg/ml ampicillin. 15 ml of the overnight culture was used to inoculate each of two 2.8-liter baffle-bottomed Fernbach flasks containing 1.5 liters of TB with 150 mg/ml ampicillin. The resulting cultures were grown at 30°C with shaking at 135 rpm to an OD_{650} of 0.5 before the addition of arabinose (0.2% (w/v)) and IPTG (200 mM). Cultures were then grown at 23°C with shaking at 135 rpm for 18 h before being harvested by centrifugation at 6000 x g for 20 min. Cell pellets were resuspended in 2 ml/g cells of lysis buffer (50 mM potassium phosphate, 150 mM NaCl, 100 mM PLP, 2 mg/ml lysozyme, 0.2 mg/ml DNase, pH 7.5) and incubated on ice for 45 min. Cells were mechanically lysed using a Constant Systems OneShot Cell Disruptor at 20 kpsi. The lysate was then clarified by centrifugation at 45000 x g for 30 min before being filtered using a 0.45 mm syringe-driven filter. Filtered lysate was then loaded onto a 5 ml HisTrap HP column (GE Healthcare). The column was washed with 3 column volumes (CV) of 100% Binding Buffer (50 mM potassium phosphate, 150 mM NaCl, 20 mM imidazole, pH 7.5), followed by 3 CV of 96% Binding Buffer and 4% Elution Buffer (50 mM potassium phosphate, 150 mM NaCl, 500 mM imidazole, pH 7.5). IlvA was eluted with a linear gradient from 4-100% Elution Buffer over the course of 10 CV. A flow rate of 2 ml/min was used to each step in protein purification. Fractions containing eluted protein were assessed for purity before being concentrated using an Amicon Ultra-15 centrifugal filter (MilliporeSigma) with a molecular weight cutoff of 30 kDa. Buffer exchange of the protein into Storage Buffer (50 mM potassium phosphate, 150 mM NaCl, 10% glycerol (v/v) pH 7.5) was performed using a PD-10 column (Cytiva). Protein concentration was determined using the Peirce BCA Protein Assay Kit (Thermo Scientific). Purified IlvA was drop frozen in liquid nitrogen and stored at -80°C until use. Purity of the protein was determined by densitometer tracing to be 94% (Figure S2).

Enzyme assays

All enzyme assays were performed at room temperature.

TABLE 3 ● Strains used in this study.

Strain	Genotype	Source
S. enterica		
DM3480	*ridA3*::MudJ[a]	Laboratory collection
DM4748	*ilvA595*::Tn10	Laboratory collection
DM6143	*ridA3*::MudJ *ilvA595*::Tn10	Laboratory collection
DM9404	Wild type	Laboratory collection
DM17845	*ridA4*::Cm[b]	This study
E. coli		
DM14520	*E. coli* K-12 MG1655 wild type (F⁻ l⁻ *rph-1*)	Laboratory collection
DM14597	*ridA790*::Km[b]	Laboratory collection
DM14669	*tdcF12*::Km[b]	Laboratory collection
DM14670	*tdcF13*::Cm[b]	Laboratory collection
DM14682	Δ*ridA890 tdcF13*::Cm[b]	Laboratory collection
DM14820	*ridA12*::Cm[b]	Laboratory collection
DM15051	Δ*ridA890* Δ *tdcF14*	Laboratory collection
DM18490	*E. coli* BL21-AI/pTEV18-*ilvA* (K12)	This study

[a] MudJ refers to the MudJ1734 transposon [42] .
[b] Denotes that gene knockouts were generated with the method described by Datsenko and Wanner [43] .

Transaminase B assay-cell extract

Cultures were grown overnight in LB (2 ml) at 37°C with shaking, and cells were pelleted and resuspended in an equal volume of sterile saline. Cell suspensions (125 μl) were used to inoculate 5 ml of the relevant medium and the resulting cultures were grown to stationary phase. Cells were then pelleted, resuspended in 10 ml of NCE medium before being pelleted and frozen at -80°C until use. Cell pellets were thawed and resuspended in 1 ml of 50 mM potassium phosphate (pH 7.5) and lysed mechanically with a Constant Systems Limited One Shot (United Kingdom) at 20 kpsi. Cell lysate was clarified by centrifugation at 17000 x g for 20 min at 4°C.

Transaminase B activity was assayed as described previously [46]. Briefly, cell free extract (50 μl) was added to a reaction mixture containing α-ketoglutarate (10 mM) in potassium phosphate buffer (50 mM, pH 7.5). The reaction was initiated with the addition of L-isoleucine (to 20 mM) and incubated at 37C for 20 minutes. Resulting 2-ketomethylvalerate (2KMV) was derivatized by 2,4-dinitrophenylhydrazine (DNPH, 200 μl) to yield a hydrazone, which was treated with 0.5 N HCl. The resulting organic layer was removed and mixed with 1.5 N NaOH to allow formation of an aqueous layer containing a chromophore with an absorbance at 540 nm. The aqueous layer was extracted, and absorbance was measured using a SpectraMax M2 microplate reader (Molecular Devices). 2KMV concentration was quantified by comparing to a standard curve, which was generated by monitoring the absorbance of reaction mixtures with known 2KMV concentrations that were subjected to the same incubation and extraction steps as described above for the cell free extracts. The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) assay (Pierce). Activity is reported as nanomoles of 2KMV per milligram of protein per cell lysate. Data plotting and statistical analyses were conducted using GraphPad Prism version 8.4.

Serine/Threonine Dehydratase assay -purified protein

The rates of 2-ketobutyrate and pyruvate formation were determined by measuring the absorbance at 230 nm [3, 39, 47] every 10 s for 2 min. Reactions were carried out in a quartz 96-well plate and measurements were acquired using a SpectraMax Plus spectrophotometer (Molecular Devices). Reaction mixtures (100 ml) contained 50 mM potassium phosphate buffer (pH 7.5), 200 nM of purified IlvA. Reactions were initiated with the addition of the indicated concentrations of L-threonine or L-serine. The absorbance at 230 nm of known concentrations of either 2-ketobutyrate or pyruvate were used to generate a standard curve to convert the change in absorbance (DA_{230}/min) to the rate of product formation (mmol product/min) for each reaction. Data visualization and statistical analyses were performed using GraphPad Prism version 8.4.

The rates of 2AA and 2AC synthesis were modeled using the equation below [39]. V_{prod} refers to the rate of product formation (2AA or 2AC) in μM/s, $k_{cat(Sub)}$ refers to the catalytic constant for $_{EC}$IlvA using the relevant substrate (s^{-1}), $[E_t]$ is the active site concentration of IlvA in E. coli, [Sub] is the concentration of substrate for $_{EC}$IlvA *in vivo* in μM, [Comp Sub] is the concentration of competing substrate in μM, $K_{M(Sub)}$ is the Michaelis-Menten constant for $_{EC}$IlvA using the relevant substrate in mM, and $K_{M(Comp Sub)}$ is the Michaelis-Menten constant for $_{EC}$IlvA using the relevant competing substrate in mM. When modeling the rate of 2AC formation, "Sub" refers to L-threonine and "Comp Sub" refers to L-serine. When modeling the rate of 2AA formation, "Sub" refers to L-serine and "Comp Sub" refers to L-threonine. 10 μM was used as the value for active site concentrations for $_{EC}$IlvA in vivo [48] and physiological concentrations of L-serine (68 μM) and L-threonine (180 μM) in *E. coli* [49], as well as elevated concentrations of L-serine (5000 μM and 20000 μM) were used when calculating the product formation by $_{EC}$IlvA.

$$Vprod = \frac{\left(k_{cat(Sub)} \times [E_t]\right)(Sub)}{K_{M(Sub)}\left(1 + \frac{[Comp\ Sub]}{K_{M\ (Comp\ Sub)}}\right) + [Sub]}$$

SUPPLEMENTAL MATERIAL

All supplemental data for this article are available online at www. microbialcell.com

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Ghatak S, King ZA, Sastry A, Palsson BO (**2019**). The y-ome defines the 35% of Escherichia coli genes that lack experimental evidence of function. **Nucleic Acids Res** 47 (5): 2446–2454. doi:10.1093/nar/gkz030

2. Niehaus TD, Gerdes S, Hodge-Hanson K, Zhukov A, Cooper AJ, Elbadawi-Sidhu M, Fiehn O, Downs DM, Hanson AD (**2015**). Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. **BMC Genomics** 16: 382. doi:10.1186/s12864-015-1584-3

3. Lambrecht JA, Flynn JM, Downs DM (**2012**). Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5′-phosphate (PLP)-dependent enzyme reactions. **J Biol Chem** 287 (5): 3454–3461. doi:10.1074/jbc.M111.304477

4. Ernst DC, Lambrecht JA, Schomer RA, Downs DM (**2014**). Endogenous Synthesis of 2-Aminoacrylate Contributes to Cysteine Sensitivity in Salmonella enterica. **J Bacteriol** 196 (18): 3335–3342. doi:10.1128/jb.01960-14

5. Ernst DC, Downs DM (**2016**). 2-Aminoacrylate Stress Induces a Context-Dependent Glycine Requirement in ridA Strains of Salmonella enterica. **J Bacteriol** 198 (3): 536–543. doi:10.1128/JB.00804-15

6. Browne BA, Ramos AI, Downs DM (**2006**). PurF-independent phosphoribosyl amine formation in yjgF mutants of Salmonella enterica utilizes the tryptophan biosynthetic enzyme complex anthranilate synthase-phosphoribosyltransferase. **J Bacteriol** 188: 6786–6792. doi:10.1128/JB.00745-06

7. Lambrecht JA, Browne BA, Downs DM (**2010**). Members of the YjgF/YER057c/UK114 family of proteins inhibit phosphoribosylamine synthesis in vitro. **J Biol Chem** 285 (45): 34,401–34,407. doi:10.1074/jbc.M110.160515

8. Bazurto JV, Farley KR, Downs DM (**2016**). An Unexpected Route to an Essential Cofactor: Escherichia coli Relies on Threonine for Thiamine Biosynthesis. **mBio** 7 (1): 1840–01,815. doi:10.1128/mBio.01840-15

9. Lambrecht JA, Downs DM (**2013**). Anthranilate phosphoribosyl transferase (TrpD) generates phosphoribosylamine for thiamine synthesis from enamines and phosphoribosyl pyrophosphate. **ACS Chem Biol** 8 (1): 242–248. doi:10.1021/cb300364k

10. Li Y, Zhang L, Luo S (**2022**). Bond Energies of Enamines. **ACS Omega** 7 (7): 6354–6374. doi:10.1021/acsomega.1c06945

11. Christopherson MR, Lambrecht JA, Downs D, Downs DM (**2012**). Suppressor analyses identify threonine as a modulator of ridA mutant phenotypes in Salmonella enterica. **PLoS One** 7 (8): 43,082. doi:10.1371/journal.pone.0043082

12. Ernst DC, Anderson ME, Downs DM (**2016**). L-2,3-diaminopropionate generates diverse metabolic stresses in Salmonella enterica. **Mol Microbiol** 101 (2): 210–223. doi:10.1111/mmi.13384

13. Fulton RL, Irons J, Downs DM (**2022**). The Cysteine Desulfurase IscS Is a Significant Target of 2-Aminoacrylate Damage in Pseudomonas aeruginosa. **mBio** 13 (3): 107,122. doi:10.1128/mbio.01071-22

14. Borchert AJ, Walejko JM, Guennec AL, Ernst DC, Edison AS, Downs DM (**2019**). Integrated Metabolomics and Transcriptomics Suggest the Global Metabolic Response to 2-Aminoacrylate Stress in Salmonella enterica. **Metabolites** 10 (1): 12. doi:10.3390/metabo10010012

15. Ernst DC, Borchert AJ, Downs DM (**2018**). Perturbation of the metabolic network in Salmonella enterica reveals cross-talk between coenzyme A and thiamine pathways. **PLoS One** 13 (5): e0197,703. doi:10.1371/journal.pone.0197703

16. Whitaker GH, Ernst DC, Downs DM (**2021**). Absence of MMF1 disrupts heme biosynthesis by targeting Hem1pin Saccharomyces cerevisiae. **Yeast** 38 (12): 615–624. doi:10.1002/yea.3670

17. Borchert AJ, Downs DM (**2017**). Endogenously generated 2-aminoacrylate inhibits motility in Salmonella enterica. **Sci Rep** 7 (1): 12,971–12,971

18. Flynn JM, Christopherson MR, Downs DM (**2013**). Decreased coenzyme A levels in ridA mutant strains of Salmonella enterica result from inactivated serine hydroxymethyltransferase. **Mol Microbiol** 89 (4): 751–759

19. Irons JL, Hodge-Hanson K, Downs DM (**2020**). RidA Proteins Protect against Metabolic Damage by Reactive Intermediates. **Microbiol Mol Biol Rev** 84 (3): 24–44. doi:10.1128/mmbr.00024-20

20. Fulton RL, Downs DM (**2023**). Modulators of a robust and efficient metabolism: Perspective and insights from the Rid superfamily of proteins. **Adv Microb Physiol** 83: 117–179. doi:10.1016/bs.ampbs.2023.04.001

21. Borchert AJ, Downs DM (**2017**). The Response to 2-Aminoacrylate Differs in Escherichia coli and Salmonella enterica, despite Shared Metabolic Components. **J Bacteriol** 199 (14): 140–157. doi:10.1128/JB.00140-17

22. Fulton RL, Downs DM (**2022**). DadY (PA5303) is required for fitness of Pseudomonas aeruginosa when growth is dependent on alanine catabolism. **Microbial Cell** 9 (12): 190–201. doi:10.15698/mic2022.12.788

23. Fulton RL, Downs DM (**2024**). Functional characterization of the dbu locus for D-branched-chain amino acid catabolism in Pseudomonas putida. **Appl Environ Microbiol** 90 (2): 196,223–196,223. doi:10.1128/aem.01962-23

24. Buckner BA, Lato AM, Campagna SR, Downs DM (**2021**). The Rid family member RutC of Escherichia coli is a 3-aminoacrylate deaminase. **J Biol Chem** 296: 100,651–100,651. doi:10.1016/j.jbc.2021.100651

25. Digiovanni S, Visentin C, Degani G, Barbiroli A, Chiara M, Regazzoni L, Pisa FD, Borchert AJ, Downs DM, Ricagno S, Vanoni MA, Popolo L (**2020**). Two novel fish paralogs provide insights into the Rid family of imine deaminases active in pre-empting enamine/imine metabolic damage. **Sci Rep** 10 (1): 10,135–10,135. doi:10.1038/s41598-020-66663-w

26. Hodge-Hanson KM, Downs DM (**2017**). Members of the Rid protein family have broad imine deaminase activity and can accelerate the Pseudomonas aeruginosa D-arginine dehydrogenase (DauA) reaction in vitro. **PLoS One** 12 (9): 185,544. doi:10.1371/journal.pone.0185544

27. Irons J, Hodge-Hanson KM, Downs DM (**2018**). PA5339, a RidA Homolog, Is Required for Full Growth in Pseudomonas aeruginosa. J Bacteriol 200 (22): 434–452. doi:10.1128/jb.00434-18

28. Irons J, Sacher JC, Szymanski CM, Downs DM (**2019**). Cj1388 Is a RidA Homolog and Is Required for Flagella Biosynthesis and/or Function in Campylobacter jejuni. **Front Microbiol** 10: 2058. doi:10.3389/fmicb.2019.02058

29. Kim KS, Pelton JG, Inwood WB, Andersen U, Kustu S, Wemmer DE (**2010**). The Rut pathway for pyrimidine degradation: novel chemistry and toxicity problems. J Bacteriol 192 (16): 4089–4102. doi:10.1128/JB.00201-10

30. Schmitz G, Downs DM (**2004**). Reduced Transaminase B (IlvE) Activity Caused by the Lack of yjgF Is Dependent on the Status of Threonine Deaminase (IlvA) in Salmonella enterica Serovar Typhimurium. J Bacteriol 186 (3): 803–810. doi:10.1128/jb.186.3.803-810.2004

31. Lambrecht JA, Schmitz GE, Downs DM (**2013**). RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life. MBio 4 (1): 33–46. doi:10.1128/mBio.00033-13

32. Shen W, Borchert AJ, Downs DM (**2022**). 2-Aminoacrylate stress damages diverse PLP-dependent enzymes in vivo. J Biol Chem 298 (6): 101,970–101,970. doi:10.1016/j.jbc.2022.101970

33. Bazurto JV, Downs DM (**2016**). Metabolic network structure and function in bacteria goes beyond conserved enzyme components. **Microbial Cell** 3 (6): 260–262. doi:10.15698/mic2016.06.509

34. Meysman P, Sanchez-Rodriguez A, Fu Q, Marchal K, Engelen K (**2013**). Expression divergence between Escherichia coli and Salmonella enterica serovar Typhimurium reflects their lifestyles. **Mol Biol Evol** 30 (6): 1302–1314. doi:10.1093/molbev/mst029

35. Datta P, Goss TJ, Omnaas JR, Patil RV (**1987**). Covalent structure of biodegradative threonine dehydratase of Escherichia coli: homology with other dehydratases. **Proc Natl Acad Sci U S A** 84 (2): 393–397. doi:10.1073/pnas.84.2.393

36. Sumantran VN, Schweizer HP, Datta P (**1990**). A novel membrane-associated threonine permease encoded by the tdcC gene of Escherichia coli. J Bacteriol 172 (8): 4288–4294. doi:10.1128/jb.172.8.4288-4294.1990

37. Hesslinger C, Fairhurst SA, Sawers G (**1998**). Novel keto acid formate-lyase and propionate kinase enzymes are components of an anaerobic pathway in Escherichia coli that degrades L-threonine to propionate. **Mol Microbiol** 27 (2): 477–492. doi:10.1046/j.1365-2958.1998.00696.x

38. Chattopadhyay S, Wu Y, Datta P (**1997**). Involvement of Fnr and ArcA in anaerobic expression of the tdc operon of Escherichia coli. J Bacteriol 179 (15): 4868–4873. doi:10.1128/jb.179.15.4868-4873.1997

39. Borchert AJ, Downs DM (**2018**). Analyses of variants of the Ser/Thr dehydratase IlvA provide insight into 2-aminoacrylate metabolism in Salmonella enterica. . J Biol Chem 293 (50): 19,240–19,249. doi:10.1074/jbc.RA118.005626

40. Ramos I, Downs DM (**2003**). Anthranilate synthase can generate sufficient phosphoribosyl amine for thiamine synthesis in Salmonella enterica. J Bacteriol 185 (17): 5125–5132. doi:10.1128/JB.185.17.5125-5132.2003

41. Davis R, Botstein D, Roth J (**1980**). Advanced bacterial genetics. Cold Spring Harbor Laboratory, New York. doi:10.1002/JOBM.19810211013

42. Castilho BA, Olfson P, Casadaban MJ (**1984**). Plasmid insertion mutagenesis and lac gene fusion with mini-mu bacteriophage transposons. J Bacteriol 158 (2): 488–495. doi:10.1128/jb.158.2.488-495.1984

43. Datsenko KA, Wanner BL (**2000**). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci 97 (12): 6640–6645. doi:10.1073/pnas.120163297

44. Boulette ML, Baynham PJ, Jorth PA, Kukavica-Ibrulj I, Longoria A, Barrera K, Levesque RC, Whiteley M (**1920**). Characterization of alanine catabolism in Pseudomonas aeruginosa and its importance for proliferation in vivo. J Bacteriol 191: 6329–6334. doi:10.1128/jb.00817-09

45. Speare L, Septer AN (**2019**). Coincubation Assay for Quantifying Competitive Interactions between Vibrio fischeri Isolates. J Vis Exp 22: 149–149. doi:10.3791/59759

46. Duggan DE, Wechsler JA (**1973**). An assay for transaminase B enzyme activity in Escherichia coli K-12. **Anal Biochem** 51 (1): 67–79. doi:10.1016/0003-2697(73)90453-3

47. Davis L (**1965**). A spectrophotometric method for the assay of threonine dehydratase. **Anal Biochem** 12 (1): 36–40. doi:10.1016/0003-2697(65)90139-9

48. Albe KR, Butler MH, Wright BE (**1990**). Cellular concentrations of enzymes and their substrates. J Theor Biol 143 (2): 80,266–80,274. doi:10.1016/s0022-5193(05)80266-8

49. Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD (**2009**). Absolute metabolite concentrations and implied enzyme active site occupancy in Escherichia coli. **Nat Chem Biol** 5 (8): 593–599. doi:10.1038/nchembio.186