

INVESTIGATING BREADTH OF THE RID PROTEIN SUPERFAMILY

by

KELSEY MARIE HODGE HANSON

(Under the Direction of Diana M. Downs)

ABSTRACT

The Rid (YjgF/YER057c/UK114) protein superfamily is ubiquitous and has been the subject of numerous structure-function efforts. The role of this superfamily remained unknown until work in bacteria elucidated a biochemical role for the RidA protein in endogenous stress response. Recent bioinformatics and phylogenetic analyses indicated the superfamily is composed of eight subgroups (RidA and Rid1-7). The archetypal member of the RidA subfamily, RidA from *Salmonella enterica*, is a reactive enamine/imine deaminase that mediates metabolic stress from the reactive metabolite 2-aminoacrylate (2AA). The majority of the structural, biochemical, and genetic data were obtained with members of the RidA subfamily. Our understanding is in the early stages for the remaining subfamilies.

The importance of RidA is reflected in the mutant phenotypes of bacteria, plants, and yeast that lack a relevant RidA homolog. Generation of 2AA, as well as the negative downstream consequences, is linked to the essential cofactor pyridoxal 5'-phosphate (PLP). Previous work has shown that 2AA deaminase activity is conserved in RidA homologs from all domains of life. This work addressed the breadth of the Rid superfamily, including biochemical characterization of Rid proteins from subfamilies Rid1, 2, and 3, that showed Rid proteins deaminate multiple substrates in addition to 2AA *in vitro*. The substrate specificities of Rid2 and Rid3 protein were

distinct from those of RidA and Rid1 subfamilies and these differences were explored by mutational analysis and biochemical analysis of variant proteins. A link was uncovered between Rid proteins and flavin adenine dinucleotide (FAD)-dependent enzymes. D-arginine dehydrogenase was identified as the third mechanism for bacterial generation of Rid substrates and this interaction was reconstituted *in vitro*. A heterologous complementation experiment identified a protein from the methanogenic archaeon *Methanococcus maripaludis* that uncovered an indirect mechanism for 2AA quenching in bacteria. Together, the ideas in this dissertation support the idea that Rid proteins facilitate deamination of reactive catalytic intermediates in multiple areas of metabolism.

INDEX WORDS: reactive metabolite, metabolic stress, Rid (YjgF/YER057c/UK114)
superfamily, bacteria

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DEDICATION

To my teachers, both formal and informal. I have been blessed with education and seemed to regularly win the luck of the draw when it came to my teachers.

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CHAPTER 1

INTRODUCTION

Cells respond to a multitude of stressors, both from external forces and endogenous sources such as reactive byproducts of metabolism. The cell must be prepared to deal with downstream consequences for any process harboring the potential to generate a harmful metabolite. Either the pathway must be used sparingly or a mediation effort must be simultaneously deployed. Members of the Rid (YjgF/YER057c/UK114) protein superfamily constitute a preemptive strategy that mediates stress caused by the reactive metabolite 2-aminoacrylate (2AA). 2AA stress has been implicated in various cellular processes in bacteria, archaea, and eukaryotes. The archetypal member of the Rid family, RidA from *Salmonella enterica*, is a reactive enamine/imine deaminase that quenches 2-aminoacrylate, which preempts damage to multiple enzymes dispersed throughout metabolism. The persistence and consequences of 2-aminoacrylate stress are both linked to the essential cofactor pyridoxal-5'-phosphate (PLP). Analyzing the functional roles of Rid proteins uncovered surprising metabolic connections, and challenged the paradigm governing metabolite diffusion and water availability within the cell.

1.1 OVERVIEW OF THE RID PARADIGM

The Rid superfamily is found in all domains of life and is composed of eight subfamilies (RidA and Rid1-7) (1). Constituents of the RidA subfamily, named for the archetypal protein RidA from *Salmonella enterica*, mediate stress that arises from the reactive metabolite 2-aminoacrylate (2AA) (2-9). Our understanding of the Rid1-7 subfamilies is in the early stages

and has been based on comparison to RidA activity and *in vitro* characterization (1, 10). All RidA proteins tested to date from bacteria, archaea, plants, humans, (11-13) and dust mites (Hodge-Hanson & Gohil, unpublished) deaminate 2AA to form pyruvate and ammonia. In the absence of a functional RidA, 2AA accumulates, inactivates certain enzymes, and leads to growth defects. Mutant phenotypes have been well characterized in *S. enterica ridA* strains (2, 3, 5-7, 9, 14, 15), and similar growth defects arise in other organisms that lack the relevant RidA homolog (1, 16-18). Therefore, the mutant phenotypes in other bacteria, plants, and yeast lacking RidA are expected to stem due from 2AA accumulation.

Multiple pyridoxal 5'-phosphate (PLP)-dependent enzymes generate 2AA as a catalytic intermediate, and a subset release the metabolite into the cellular milieu, including the bacterial enzymes threonine/serine dehydratase (EC 4.3.1.19), cysteine desulfhydrase (EC 4.4.1.15), and diaminopropionate ammonia-lyase (EC 4.3.1.15). Free 2AA has one of four possible fates: i) free 2AA inactivates particular PLP-enzymes, ultimately leading to growth defects, ii) 2AA is spontaneously quenched by solvent water, iii) 2AA is quenched via deamination by RidA, or iv) 2AA persists in the cellular milieu and may react with other cellular components (Figure 1.1). In the presence of a functional RidA, the first fate is minimized and sensitive PLP-enzymes are spared. The mutant phenotypes that arise in the absence of a functional RidA indicate that the rate of the second fate is not sufficient to protect the cell from 2AA stress, and RidA is required to restore metabolic balance. *In vitro*, addition of purified RidA accelerated the rate of pyruvate formation from purified 2AA-releasing enzymes (7, 9, 11) This was an important point, which indicated solvent water limited 2AA quenching in aqueous solution and suggested that the reaction would occur more slowly in the cell where water availability is lower (19-21). Relevance of the fourth fate is supported by the existence of at least two RidA-independent 2AA

quenching mechanisms that are consistent with an indirect deamination mechanism (Hodge-Hanson, unpublished; Ernst & Christopherson, unpublished). It is possible that this mechanism entails either an enzyme with moonlighting deaminase activity or is the result of metabolite-metabolite interactions.

The *in vivo* characterization of *S. enterica ridA* mutant strains combined with *in vitro* biochemistry informed the RidA paradigm and challenged the idea that short-lived intermediates in PLP-dependent reactions would be inconsequential *in vivo*. *In vitro*, RidA facilitated an increase in the overall rate of the reaction for three separate enzymes, despite an excess of solvent water (7, 9, 11). This raised an interesting question: does the reaction with or without RidA more closely reflect the rate of the pathway *in vivo*? This ushered into consideration the first committed step of *S. enterica* isoleucine biosynthesis in which threonine/serine dehydratase IlvA generates 2-ketobutyrate from threonine. In addition to accelerating the formation of pyruvate from 2AA, RidA accelerated 2-ketobutyrate formation from 2-aminocrotonate (2AC) (21). While it is not required for isoleucine biosynthesis, it is likely that RidA contributes to 2-ketobutyrate formation *in vivo*. Although *S. enterica ridA* strain growth defects are rescued by isoleucine supplementation, previous work concluded that the relevant mechanism is allosteric inhibition of 2AA generation by IlvA rather than an isoleucine limitation (5, 22). In contrast to the extensive damage caused by 2AA, there is no indication that 2AC has negative consequences. Given that available water can catalyze identical deamination of metabolites such as 2AC that are not expected to precipitate metabolic stress, we anticipate that any phenotypic effects of these metabolites will be subtle in the absence of RidA.

Imines are produced as catalytic intermediates by FAD-dependent oxidases and dehydrogenases that convert an amino acid ultimately to a ketoacid. The reaction is reminiscent

of the PLP-dependent dehydratases and the two mechanisms are compared in Figure 1.2. *In vitro*, purified Rid2 and Rid3 proteins accelerated the overall rate of the D-arginine dehydrogenase (*Pseudomonas aeruginosa* DauA) reaction (10). Based on these observations, it is possible that Rid proteins might serve an analogous role in additional pathways that rely on spontaneous reaction with water. FAD- and PLP-enzymes are often encoded in putative operons with Rid proteins in *Pseudomonas aeruginosa* and other species. These observations suggested RidA and Rid1-7 proteins might serve distinct cellular roles and were consistent with the idea that Rid proteins are employed as a mediation tactic for pathways involving reactive metabolites.

1.2 DISSERTATION OUTLINE

This work explored the breadth of the Rid protein superfamily using biochemical and genetic approaches. Previous work on RidA from *Salmonella enterica* established the framework of the Rid paradigm, which described the 2AA stress response role of Rid proteins. Beyond the RidA subfamily, I biochemically characterized diverse bacterial Rid enzymes from subfamilies 1, 2, and 3, and probed substrate specificity. I also identified the third mechanism for *in vivo* generation of a Rid substrate and reconstituted the reaction *in vitro*.

Chapter 2 discusses the biochemical activities associated with members of the Rid protein superfamily. This literature review provides a comprehensive view of Rid superfamily members, defines RidA activity, and highlights the disparity of Rid1-7 subfamilies. Three recently solved crystal structures of Rid1 and Rid2 proteins were compared to the numerous existing RidA structures that, along with previous biochemical data, supported the hypothesis that the arginine residue was necessary and sufficient for activity. Finally, this chapter addresses a functional annotation quandary that has arisen as additional genomes are sequenced.

In Chapter 3, I showed Rid2 and Rid3 proteins accelerate the D-arginine dehydrogenase (*Pseudomonas aeruginosa* DauA) reaction *in vitro*, identifying the third bacterial mechanism that generates substrates for Rid proteins. The distinction between RidA and Rid1 versus Rid2 and Rid3 was the first biochemical separation between active deaminases.

Chapter 4 describes a heterologous complementation approach that identified a putative aspartate/glutamate racemase from a methanogenic archaeon with the ability to substitute for *S. enterica* RidA *in vivo*. This suggested metabolic networks might be configured to indirectly quench 2AA in contrast to the RidA mechanism of direct deamination.

In Chapter 5, I demonstrate that a growth defect arises in *P. aeruginosa* mutant strains lacking RidA homolog PA5339. The mutant phenotype was dissected with nutritional analysis and complementation studies. *P. aeruginosa* encodes three RidA homologs in addition to PA5339 and multiple non-RidA subfamily proteins, which provided a platform to explore genomic redundancy and probe regulation.

In Chapter 6, I probed the mechanism of iminoarginine deaminase activity. Along with the crystal structure of *P. aeruginosa* PA5083, the biochemical data are consistent with a model where the critical active site arginine residue is required for deaminase activity.

Chapter 7 summarizes the conclusions from my work, and outlines future directions for the study of Rid proteins.

Appendix A explores the *S. enterica* *ridA* mutant strain sensitivity to perturbations in arginine/pyrimidine metabolism. The findings presented here suggested RidA might be connected to carbamoyl phosphate metabolism, but it was not clear whether 2AA was ultimately the metabolite responsible for the growth defects.

REFERENCES

1. Niehaus TD, Gerdes S, Hodge-Hanson K, Zhukov A, Cooper AJ, ElBadawi-Sidhu M, et al. Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC Genomics*. 2015;16:382.
2. Enos-Berlage JL, Langendorf MJ, Downs DM. Complex metabolic phenotypes caused by a mutation in yjgF, encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of bacteriology*. 1998;180(24):6519-28.
3. Browne BA, Ramos AI, Downs DM. PurF-independent phosphoribosyl amine formation in yjgF mutants of *Salmonella enterica* utilizes the tryptophan biosynthetic enzyme complex anthranilate synthase-phosphoribosyltransferase. *Journal of bacteriology*. 2006;188(19):6786-92.
4. Lambrecht JA, Browne BA, Downs DM. Members of the YjgF/YER057c/UK114 family of proteins inhibit phosphoribosylamine synthesis in vitro. *The Journal of biological chemistry*. 2010;285(45):34401-7.
5. Christopherson MR, Schmitz GE, Downs DM. YjgF is required for isoleucine biosynthesis when *Salmonella enterica* is grown on pyruvate medium. *J Bacteriol*. 2008;190(8):3057-62.
6. Christopherson MR, Lambrecht JA, Downs D, Downs DM. Suppressor analyses identify threonine as a modulator of ridA mutant phenotypes in *Salmonella enterica*. *PloS one*. 2012;7(8):e43082.
7. Ernst DC, Lambrecht JA, Schomer RA, Downs DM. Endogenous synthesis of 2-aminoacrylate contributes to cysteine sensitivity in *Salmonella enterica*. *Journal of bacteriology*. 2014;196(18):3335-42.
8. Downs DM, Ernst DC. From microbiology to cancer biology: the Rid protein family prevents cellular damage caused by endogenously generated reactive nitrogen species. *Molecular microbiology*. 2015;96(2):211-9.
9. Ernst DC, Anderson ME, Downs DM. L-2,3-diaminopropionate generates diverse metabolic stresses in *Salmonella enterica*. *Molecular microbiology*. 2016;101(2):210-23.
10. Hodge-Hanson KM, Downs DM. 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*. 2017;12(9):e0185544.
11. Flynn JM, Christopherson MR, Downs DM. Decreased coenzyme A levels in ridA mutant strains of *Salmonella enterica* result from inactivated serine hydroxymethyltransferase. *Molecular microbiology*. 2013;89(4):751-9.

12. Flynn JM, Downs DM. In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5'-phosphate cofactor. *Journal of bacteriology*. 2013;195(16):3603-9.
13. Leitner-Dagan Y, Ovadis M, Zuker A, Shklarman E, Ohad I, Tzfira T, et al. CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. *Planta*. 2006;225(1):89-102.
14. Oxelmark E, Marchini A, Malanchi I, Magherini F, Jaquet L, Hajibagheri MA, et al. Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome. *Molecular and cellular biology*. 2000;20(20):7784-97.
15. Kim JM, Yoshikawa H, Shirahige K. A member of the YER057c/yjgF/Uk114 family links isoleucine biosynthesis and intact mitochondria maintenance in *Saccharomyces cerevisiae*. *Genes to cells : devoted to molecular & cellular mechanisms*. 2001;6(6):507-17.
16. Lambrecht JA, Schmitz GE, Downs DM. RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life. *mBio*. 2013;4(1):e00033-13.
17. Kreuzer-Martin HW, Lott MJ, Ehleringer JR, Hegg EL. Metabolic processes account for the majority of the intracellular water in log-phase *Escherichia coli* cells as revealed by hydrogen isotopes. *Biochemistry*. 2006;45(45):13622-30.
18. Zhou HX, Rivas G, Minton AP. Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu Rev Biophys*. 2008;37:375-97.
19. Lambrecht JA, Flynn JM, Downs DM. Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions. *J Biol Chem*. 2012;287(5):3454-61.
20. Schmitz G, Downs DM. 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. *Journal of bacteriology*. 2004;186(3):803-10.
21. Niehaus TD, Nguyen TND, Gidda SK, ElBadawi-Sidhu M, Lambrecht JA, McCarty DR, et al. Arabidopsis and Maize RidA Proteins Preempt Reactive Enamine/Imine Damage to Branched-Chain Amino Acid Biosynthesis in Plastids. *The Plant Cell*. 2014;26(7):3010-22.
22. Li C, Lu CD. Arginine racemization by coupled catabolic and anabolic dehydrogenases. *Proc Natl Acad Sci U S A*. 2009;106(3):906-11.

23. Yuan H, Fu G, Brooks PT, Weber I, Gadda G. Steady-state kinetic mechanism and reductive half-reaction of D-arginine dehydrogenase from *Pseudomonas aeruginosa*. *Biochemistry*. 2010;49(44):9542-50.
24. Yuan H, Xin Y, Hamelberg D, Gadda G. Insights on the mechanism of amine oxidation catalyzed by D-arginine dehydrogenase through pH and kinetic isotope effects. *J Am Chem Soc*. 2011;133(46):18957-65.

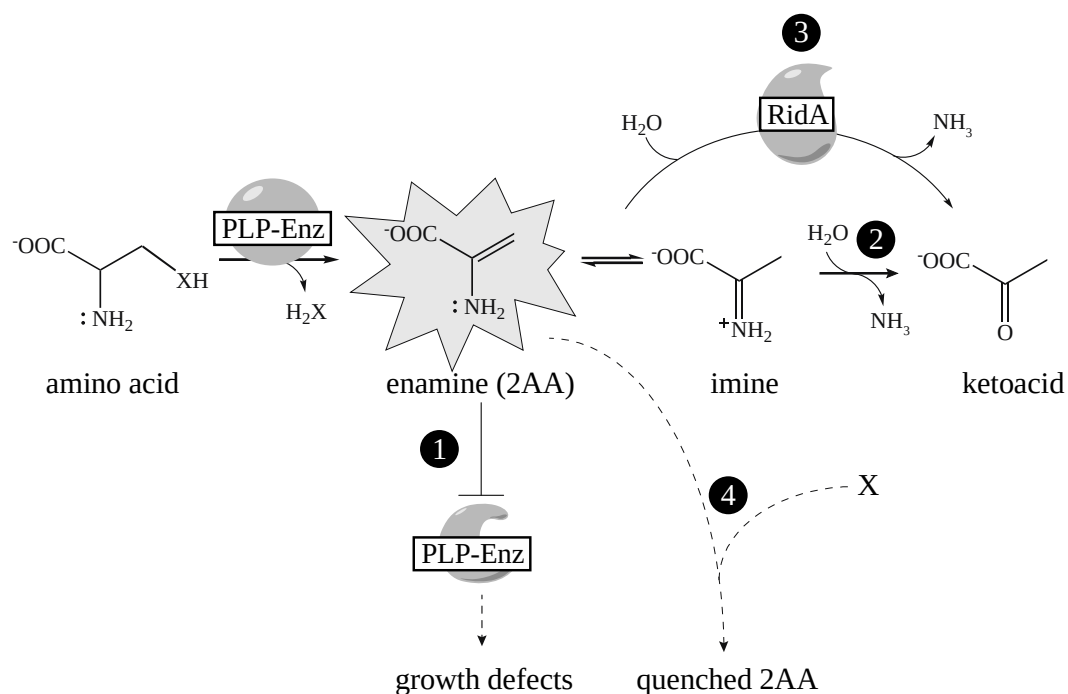
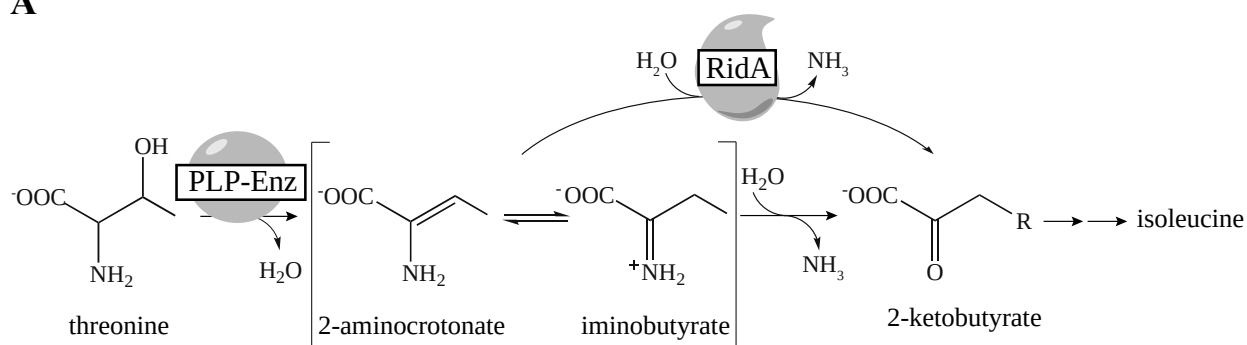
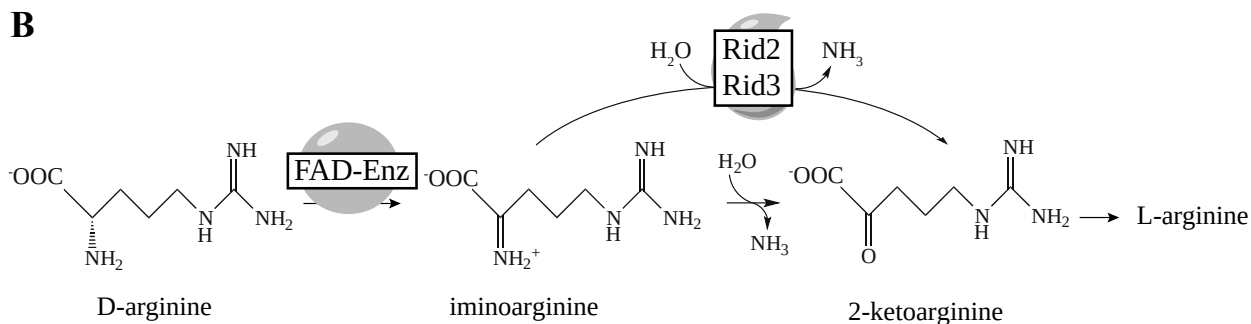


Figure 1.1 The RidA paradigm

Certain PLP-dependent enzymes generate 2-aminoacrylate 2AA as a catalytic intermediate. 2AA that is released into the cellular milieu meets one of the following fates: 1) free 2AA inactivates particular PLP-enzymes which ultimately leads to growth defects in the absence of a functional RidA, 2) following spontaneous tautomerization to the imine species, the reactive metabolite is spontaneously deaminated by water to form the ketoacid pyruvate 3) RidA directly deaminates 2AA, releasing ammonia and pyruvate, or 4) 2AA may be quenched via an indirect mechanism represented in the diagram as “X”. Abbreviations: PLP (pyridoxal 5'-phosphate); Enz (enzyme); 2AA (2-aminoacrylate).

A**B****Figure 1.2 Bacterial pathways that generate Rid substrates**

(A) The first committed step in isoleucine biosynthesis is conversion of threonine to 2-aminocrotonate by PLP-dependent threonine/serine dehydratase. 2-aminocrotonate (enamine) tautomerizes to iminobutyrate (imine), and is then deaminated spontaneously by water to produce 2-ketobutyrate, the subsequent metabolite in the pathway. RidA accelerates the rate of this reaction *in vitro* and *in vivo* (5, 21, 23). (B) D-arginine dehydrogenase DauA from *Pseudomonas aeruginosa* catalyzes an analogous reaction in arginine metabolism (24-26). Rid2 and Rid3 proteins accelerated iminoarginine (imine) deamination *in vitro*.

CHAPTER 2

LITERATURE REVIEW OF THE BIOCHEMICAL ACTIVITIES ASSOCIATED WITH RID
PROTEINS¹

¹ Hodge-Hanson, K. M, D. Zhou, D. M. Downs, 2017. *To be submitted to FEMS Microbiology Reviews*

2.1 ABSTRACT

The Rid (YjgF/YER057c/UK114) protein superfamily is conserved in all domains of life and composed of eight subfamilies (RidA, and Rid1-7) (1). Members of the Rid family have been implicated in myriad cellular processes including amino acid biosynthesis, respiration, development, carcinogenesis and allergies. In addition, mutant phenotypes arise in multiple organisms that lack the relevant Rid, and the evidence suggests damage by the reactive metabolite 2-aminoacrylate (2AA) is responsible for these growth defects. In addition to 2AA deaminase activity, *in vivo* and *in vitro*, multiple biochemical activities have been associated with Rid proteins, and localization studies indicate where these proteins might function in different types of cells. This review focuses on the best-characterized Rid subfamily (RidA), highlighting the experimental evidence that led to the identification of the first functional role within the Rid superfamily.

2.2 INTRODUCTION

Members of the Rid (YjgF/YER057c/UK114) superfamily were recognized early in all domains of life based on sequence homology and their ubiquity suggests an ancient and essential role. The first crystal structure (*Escherichia coli* RidA) was solved in 1999 (2). This finding spurred additional structure-function efforts, which resulted in over twenty crystal structures of bacterial or eukaryotic RidA homologs, many of which were deposited to the Protein Data Bank without accompanying publications (Table 2.1). All Rid proteins to date are homotrimeric and share a chorismate mutase fold type, although varying degrees of compaction may influence interpretation of the true oligomeric state (3). The mechanism was proposed based on the residues that were strictly conserved among the sequences of Rid proteins identified at the time (4, 5). However, as sequence data amassed, exceptions were identified in the previously

considered “invariant” residues, and it became clear that Rid protein sequences were highly diverse surrounding the active site (1).

Rid subfamilies

Phylogenetic and bioinformatics analyses that included more than 1,000 bacterial genomes guided division of the superfamily into eight subfamilies (RidA and Rid1-7) (1). RidA proteins were present in all three domains, while Rid1-7 proteins were limited to prokaryotes. Table 2.2 shows the distribution of Rid proteins in selected bacterial genomes. Some bacterial genomes encoded up to sixteen Rid proteins, and typically included one or more RidA proteins, as well as other Rids that spanned additional subfamilies. These observations suggested RidA might be a housekeeping enzyme and the Rid1-7 proteins may serve more specialized roles.

A superfamily of unknown function

The functional role of RidA was characterized in *Salmonella enterica*, and was dissected through biochemical and genetic experiments. Our understanding of the remaining subfamilies is in its infancy. The rate of functional annotation lags behind the number of genes sequenced, such that genes of unknown function are likely to be misannotated or to lack annotation (1, 6, 7). The Rid superfamily illustrates a quandary about functional analyses that resonates throughout the biological sciences. It is important to note that elucidation of the RidA paradigm required broad experimentation coupled with serendipity, and it is doubtful that a singular approach would have resulted in functional role characterization. This review summarizes the progress that has been made in understanding the biochemical activity and physiological role of members of the Rid superfamily.

Synteny

Genes encoding Rid proteins cluster with potential enamine/imine generating enzymes that use either PLP or FAD as a cofactor (1). For example, the *Pseudomonas aeruginosa* genome encodes nine Rid proteins, including multiple loci near probably FAD-dependent oxidases. Biochemical and genetics studies have shown that RidA accelerated the rate of a PLP-dependent step in isoleucine biosynthetic pathway (8, 9), and Rid2 and Rid3 proteins accelerated the rate of an FAD-dependent step in D-arginine metabolism (10). These data and the genomic synteny observations are consistent with a model where Rid proteins evolved to mediate steps involving certain reactive metabolites.

2.3 VALIDATED RID DEAMINASES

Definition of Rid activity

RidA, is a reactive enamine/imine deaminase, a function shared by every RidA tested to date, including *Pyrococcus furiosus* PF0668 (11), *Cucumis sativus* ChrD (11), *Homo sapiens* UK114 (11), *Arabidopsis thaliana* (12), *Zea mays* (12), *Dermatophagoides farina* (house dust mite) (13), and *Escherichia coli* RidA (14) (Table 2.3). A functional role for a protein is best demonstrated by a mutant phenotype in its absence. In the absence of a RidA protein, mutant phenotypes arise in bacteria, plants, and yeast, indicating that RidA affects diverse metabolic processes such as nutrition, mitochondrial maintenance, 1-carbon metabolism, and photosynthesis (Figure 2.1). Therefore, it is likely that described and emerging mutant phenotypes in other organisms ultimately stem from 2AA accumulation.

Summary of the RidA paradigm

The mechanisms at play were best described by work in *S. enterica*, which established the 2AA stress response RidA paradigm (8, 15-18) (Figure 2.2). 2AA is generated as a catalytic

intermediate by various fold type II pyridoxal 5'-phosphate (PLP)-dependent enzymes (19-28), including threonine/serine dehydratase (EC 4.3.1.19), cysteine desulfhydrase (EC 4.4.1.15), and diaminopropionate ammonia-lyase EC 4.3.1.15), which release 2AA into the cellular milieu (9, 29-31) (Figure 2.3). Free 2AA meets one of three fates i) diffusion throughout the cell and reaction with various enzymes that results in inactivation ii) tautomerization to the imine species followed by spontaneous deamination by water, or iii) enzymatic deamination by RidA. Deamination of 2AA leads to the production of pyruvate, a less reactive molecule, such that RidA activity increases pyruvate formation and simultaneously lowers the intracellular pool of reactive 2AA. The enzymes inactivated by 2AA are PLP-dependent enzymes, including isoleucine transaminase (EC 2.6.1.42) (11, 32), alanine racemase (EC 5.1.1.1) (33), serine hydroxymethyltransferase (EC 2.1.2.1) (34), and aspartate aminotransferase (EC 2.6.1.1) (14). In each of these cases, specific activity was decreased in a bacterial *ridA* mutant strain experiencing 2AA stress. Isoleucine transaminase was also inactivated in yeast strains that lacked the relevant RidA homolog (35). The PLP-enzymes that are either 2AA generators or targets of 2AA damage can be found in all three domains. *In vitro* assays for 2AA release or 2AA inactivation suggested that the PLP-dependent enzyme mechanisms described in bacteria are universal (11, 12)(Ernst, 2017 submitted).

Rid1-7 subfamilies

Our understanding of the Rid1-7 subfamilies is in the early stages. Biochemical surveys and solved structures have begun to probe the roles of Rid1-3 proteins. Members of subfamilies Rid4-7 lack the active site arginine residue required for deamination and are therefore not expected to share function with other Rid proteins. To date, only one mutant phenotype with rigorous complementation data has been described in the absence of a non-RidA subfamily

member. *Campylobacter jejuni* strains lacking Cj1388 (Rid2) have decreased agglutination, reduced motility, and increased sensitivity to oxidative stress or low pH (36-38). These observations support the idea that non-RidA proteins might play specialized cellular roles. Studying additional mutant phenotypes will guide elucidation of these activities.

Table 2.3 shows validated Rid1-3 deaminases that acted on the enamine 2AA as well as a broad range of imine intermediates *in vitro* (1, 10). However, it was not clear which imines would be relevant *in vivo*. If non-RidA proteins serve specialized roles, then Rid1-3 proteins might participate in metabolic pathways that produce imines from various amino acids. In particular, Rid2 and Rid3 proteins deaminated iminoarginine and ultimately facilitated the overall rate of a single step in D-arginine metabolism (10). The *in vivo* relevance has yet to be explored for the imine derivatives of leucine, phenylalanine, glutamine, methionine, or histidine, which were all deaminated by Rid proteins *in vitro*. In contrast to 2AA, the evidence seems to suggest that the intermediate 2-aminocrotonate (2AC) was unable to elicit an endogenous stress response. 2AC has one additional methyl group in comparison to 2AA and yet did not impede growth of a *ridA* mutant strain nor damage 2AA-susceptible enzymes *in vitro*. Rid proteins deaminated both 2AC and 2AA and both metabolites accumulate in a *ridA* mutant strain (17, 39). Given these observations, it is likely that accumulation of certain enamine/imine intermediates will not lead to 2AA-like metabolic stress. Rid proteins might deaminate enamine/imine intermediates in order to facilitate efficient metabolism in the cell, where water availability is low (9, 40, 41).

Deaminase mechanism

The Rid active site is nestled between subunits and the cleft binds water in close proximity to the substrate (4, 5). Although many residues that line the active site appear to be

conserved in multiple Rid sequences, a single arginine residue is necessary and sufficient to bind the substrate (1). Figure 2.4 shows the interaction between *S. enterica* RidA Arg105 and the imine carboxyl group. Variant protein activity indicated that residues Cys107, Tyr17, and Glu120 were dispensable and supported the conclusion that Arg105 alone was critical (9). Additional residues have been shown to impact overall protein structure (42). Recently solved structures of non-RidA proteins PA0814 (Rid1), PSPTO_0102 (Rid2) and PA5083 (Rid2) showed overall active site architecture similar to RidA (Figure 2.4). Crystal structures with ketoacids soaked into the active site such as TdcF with serine (5) or PA5083 with 2-ketobutyrate provide the basis for our understanding of the enzyme-substrate interaction. First, water is bound in the active site by amino acid backbones, rather than functional groups. This in part explains the residue diversity in the active site pocket of active Rid deaminases. Second, if the critical enzyme-substrate interaction occurs at the carboxyl end, the broad substrate specificity of Rid enzymes is plausible.

2.4 BIOCHEMICAL ACTIVITIES ASSOCIATED WITH RIDA PROTEINS

The identification of a ubiquitous superfamily of unknown function spurred broad interest in biochemical characterization of Rid proteins from the three domains. As a result, the collection of Rid superfamily knowledge includes localization studies done on many cell types, information on expression patterns, and reports of protein modifications that might influence activity in the cell. In order to assimilate these currently disparate activities, it is critical to evaluate which are physiologically relevant. As the current and emerging mutant phenotypes that arise in the absence of relevant RidA proteins are expected to be sequelae of 2AA stress, we present the RidA biochemical activities in this light. Every RidA homolog tested to date catalyzes 2AA deamination and therefore, is the only biochemical activity that unifies proteins

across domains. It is critical to establish whether 2AA deamination is relevant to the mechanism of any additional activity in order to establish whether Rid proteins might serve moonlighting functions that are distinct from 2AA stress. As our understanding of the Rid superfamily progresses, we reflect on associations reported in the literature to establish a consensus view of the Rid superfamily.

Translation inhibitor

Eukaryotic RidA proteins were originally designated as perchloric acid-soluble proteins (PSP) with endoribonuclease activity. Crude extracts or purification fractions containing RidA (formerly PSP) proteins from rat liver (43, 44) or human monocytes (45) inhibited translation in cell-free assays. Furthermore, this effect on translation was eliminated in the extracts following heat treatment or precipitation via RidA-specific antibodies. Endoribonuclease activity could provide a mechanism for inhibiting translation. Together, UK114 and a Y-box protein from human monocytes degraded mRNA, however the recombinant protein did not retain this activity (46). A fraction that contained native RidA from *Rhodopseudomonas palustris* (Rhp-PSP) and the recombinant protein demonstrated antiviral properties on tobacco mosaic virus infection of tobacco leaves (47), suggesting attractive areas of potential application for members of the Rid superfamily.

It is important to consider whether other proteins, or non-protein cellular components, might associate with RidA and account for the translation inhibitor properties of a protein purification fraction. This might explain discrepancies between native and recombinant proteins. It is formally possible that RidA catalyzes both deamination, requiring an active site arginine, and a secondary endoribonuclease activity that results in decreased translation under the conditions tested. Construction of variant proteins at the critical active site residue might

simultaneously resolve whether an active RidA deaminase is required and could confirm whether RidA is responsible for the inhibitory effect on translation.

Localization of RidA proteins

High-throughput studies have identified RidA homologs in multiple types of cells. RidA (PSP) protein was found in various eukaryotic tissues including the lung (48), digestive tract (49), liver (43, 44, 50-52), kidney (53), white blood cells (45, 46), and oocytes (54). Changes in intracellular protein localization were correlated with treatment of rat kidney tissue with various compounds that elicit endoplasmic reticulum stress (53). In plant cells, RidA was localized to the chloroplasts (12, 55) and in yeast the RidA homolog Mmf1p was found in the mitochondria (56).

The broad complementation of *S. enterica* *ridA* mutant strains with diverse homologs from the three domains seemed to suggest that interaction with another protein is likely not required. It is possible that RidA interacts with other cellular components, and the extent to which any putative interactions influence activity should be explored. A protein interaction screen in *Helicobacter pylori* identified a connection between RidA homolog HP0944 and DnaA (57). In bacteria, RidA proteins lack secretory tags and are predicted to remain in the cytosol (58), however there is some evidence to suggest RidA may be relevant outside of the cell. The RidA homolog MCR_0348 appeared to be required for *Moraxella catarrhalis* attachment to human epithelial cells (59).

In two scenarios, antibodies to a RidA protein are linked to human disease. The human RidA protein UK114 has been implicated in tumor growth, and UK114-specific antibodies seemed to have inhibitory properties (60-62). The connections between 2AA stress and cancer biology have been discussed elsewhere (31). Recently, a connection was established between the RidA protein (Der f 34) from the house dust mite (*Dermatophagoides farinae*) and human

allergies (13). Human development of antibodies to an allergen protein (RidA) implies that RidA might be actively translocated by a type of dust mite secretory mechanism or released along with all cytosolic contents following cell lysis.

If RidA proteins catalyze multiple activities, differences in localization might contribute to the relevant activity in each scenario. For instance, 2AA deaminase activity may or may not be required extracellularly if the metabolite is not generated, whereas the relevance of this role *in vivo* has been established. Studies on the possible roles of extracellular RidA might seek the critical portions of the protein, and test 2AA deaminase null variants to probe the connection to 2AA stress in this context.

Expression or abundance of RidA proteins

A number of studies found patterns in Rid expression that were correlated with various cellular processes, and provide incentive for additional experimentation to test potential Rid connections to particular metabolites. Most of the expression or protein abundance data correspond to members of the RidA subfamily. This was not surprising, given i) the earliest described superfamily members belong to the RidA subfamily, and ii) RidA is the only subfamily found in eukaryotes. Eukaryotic studies suggested RidA homolog expression could be linked to cell differentiation (45), healthy cell proliferation (63, 64), fasting (65), meiosis in oocytes (54), or *Giardia duodenalis* host attachment (66), and that expression might be influenced by promoter methylation (52).

The use of high-throughput approaches to profile bacterial transcription fortuitously uncovered multiple Rid expression patterns. Expression of a Rid protein was associated with biofilm formation in *E. coli* (67) and *Salmonella enterica* serovar Enteritidis (68). Other correlations have been drawn between Rid expression and growth at higher temperatures

(*Clostridium difficile* (69)), amino acid metabolism (*Pseudomonas aeruginosa* (70) and *Streptococcus pyogenes* (71)), or agmatine/putrescine metabolism (*Pseudomonas aeruginosa* (72)). A separate RidA homolog in *Streptococcus pyogenes*, seemed to be upregulated in strains with a defective two-component regulatory system (73). The effect of a *ridA* mutation on the transcriptional profile in a cell has been probed in bacteria and yeast (52, 74). Taken together, these observations support the idea that RidA affects diverse metabolic processes in all domains of life.

Metabolism of aromatic compounds or antibiotics

Analogous to the formation of 2AA in isoleucine biosynthesis, pathways elsewhere in metabolism generate similar enamine/imine intermediates via PLP- or FAD- dependent enzymes. Other intermediates may be released into the cellular milieu and could serve as Rid substrates, as was recently described for the FAD-dependent dehydrogenase DauA (10). Compelling gene clusters and/or biochemical evidence suggests Rid proteins might function in antibiotic biosynthesis pathways (75) or in degradation of aromatic compounds (76-81). These studies present exciting opportunities to examine the impact of non-2AA enamine/imine intermediates on bacterial metabolism.

Modified RidA proteins

In some cases, isolation of a RidA protein suggested the enzyme might be modified *in vivo*. Reports indicated cleavage of the initial RidA methionine from *E. coli* cultures (82, 83). In human liver cells, RidA homolog p14.5 DNA methylation was associated with aberrant proliferation, which was reminiscent of the mechanism used to silence tumor suppressing genes (52). *In vitro*, the mouse liver homolog was phosphorylated by a phosphate kinase (51). A lipoprotein probe extracted a bacterial RidA protein from *E. coli* lysate and suggested that the

Cys107 residue might be capable of binding fatty acids (84). This residue was also modified following peroxynitrite exposure (85). These modifications raise a number of questions regarding the physiological relevance. While the Cys107 residue is located within the RidA active site pocket, alanine substitutions to this residue failed to abolish activity *in vivo* or *in vitro*, which indicated the residue was not required for 2AA stress response activity (9). Despite similar 2AC deaminase activity *in vitro*, a RidA^{C107S} variant retained activity following peroxynitrite exposure in contrast to the inhibition observed with the wild type protein (85). This discrepancy was surprising, and the authors proposed the variant was constitutively active yet “redox-dead”.

Peroxynitrite can be spontaneously generated from nitric oxide and superoxide, and it is likely that bacteria would encounter this reactive molecule. *E. coli ridA* mutant strains were more sensitive to peroxynitrite compared to wild type (85). Complementation data would bolster support for the conclusion that the mutation in *ridA* was causative. If an allele encoding RidA^{C107S} restores growth *in trans*, then these experiments would support the hypothesis that the variant protein is insensitive to peroxynitrite. The peroxynitrite experiments were consistent with the data that showed deaminase activity is unaffected by modifications to Cys107. As it is possible that RidA performs a separate function in the cell, in addition to its role as a 2AA deaminase, future studies might delineate these two functions in a physiological context. Given the differences between the metabolic networks of closely related organisms such as *Salmonella enterica* and *Escherichia coli* (14), the redox phenotypes of *ridA* mutant strains in other organisms might be explored.

Chaperone (or heat shock protein) activity

Early sequence alignments suggested that certain RidA subfamily proteins were homologous to calpain-activating proteins (86) or heat shock proteins (51), which prompted

assessments of chaperone activity. However, these biochemical activities were protein-specific. Protein fractions of human RidA (UK114) and additional cell components catalyzed calpain activation (86), however, this activity was not detected with purified *Drosophila melanogaster* RidA (DUK114) (87). In contrast, DUK114 seemed to be an active chaperone in *in vitro* citrate synthase assays (87). The sequence similarity between rat RidA (P23) and a heat shock protein (88) was not observed in similar comparisons including human p14.5 (45) or bacterial RidA proteins. Subsequent alignments and phylogenetic studies led to the identification of diverse Rid superfamily members (1) and supported the idea that the ubiquitous superfamily does not universally share homology to heat shock proteins. The extensive annotation of RidA proteins as putative heat shock proteins was perpetuated from a small number of early sequence alignments. Here we discuss historical annotations and assimilate the RidA subfamily data from broad biological fields into a comprehensive model that will guide future RidA experimentation in organisms from all domains of life.

Based on the data that suggest mutant phenotypes manifest as a result of 2AA stress in bacteria, plants and yeast (11, 12, 33, 34, 55, 78, 89, 90), any phenotypes that might arise independent of 2AA are exciting. *E. coli ridA* strains appear to be more sensitive to peroxynitrite or hypochlorite (HOCl) compared to wild type (85, 91). However, 2AA has been shown to indirectly damage multiple nodes of the metabolic network (30, 34) and it is not clear at this point whether these phenotypes arise independent of the permeating effects of 2AA. It remains possible that the growth defect ultimately stems from an inactivated PLP-enzyme, and perhaps a new 2AA-susceptible enzyme might be identified. We propose a series of experiments to establish whether emerging phenotypes ultimately stem from 2AA stress. First, if growth defects arise independently of 2AA, growth will not be improved by supplementation with glycine

and/or isoleucine, nutrients that halt 2AA generation (isoleucine) or circumvent 2AA-damaged enzymatic steps (glycine). Second, if the effects are 2AA-independent, specific activities of 2AA-sensitive enzymes (threonine/serine dehydratase, alanine racemase, serine hydroxymethyltransferase and aspartate aminotransferase) are expected to match wild type levels. Screening additional PLP-enzymes in fold-type I, III, and IV families might rule out the alternative hypothesis that involves inhibition of a novel 2AA target. Decreased activity of any combination of these enzymes suggests the growth defect ultimately stems from 2AA stress. Any phenotypes associated with loss of RidA are excellent candidates for additional supplementation studies that might reveal areas of metabolism damaged by the relevant metabolite (i.e. HOCl, or peroxynitrite). There is evidence that the 2AA response differs between closely related organisms *E. coli* and *S. enterica* (14) and given that a second RidA homolog (TdcF) exists in *E. coli*, it was interesting that a HOCl-induced growth defect was elicited by the sole mutation in *ridA* (91). The role of TdcF in the context of peroxynitrite or HOCl stress remains unclear.

In a similar vein it is important to consider whether stressors such as peroxynitrite or HOCl elicit a similar response in other organisms that lack the relevant RidA homolog. The data from a single seminal study by Muller, *et al.* has been cited in multiple review articles (92, 93). Here, we assimilate the HOCl experiments and bacterial 2AA stress paradigm. The sensitivity of a *ridA* strain to HOCl was evaluated in rich medium using a popular protein purification strain (94). If a *ridA* strain exhibits HOCl sensitivity in a buffered minimal medium, this would provide an opportunity to test the effects of isoleucine and glycine on the growth defect in order to address the possible implication of 2AA and to identify pathways affected by HOCl damage.

In vitro, HOCl-modified RidA (RidA^{HOCl}) inhibited the rate of 2-ketobutyrate formation from IlvA with threonine (91). In addition to the interpretation that RidA^{HOCl} and IlvA directly

interact, additional scenarios are possible. For instance, stability of the HOCl modification to RidA remains unclear. If the modification is transient, then release of HOCl from RidA might permit interaction with IlvA or the substrate and could explain the loss of activity. Isolating the RidA^{HOCl} protein from *E. coli* following exposure to HOCl might provide insight on the stability of the modification and provide evidence for the modification *in vivo*.

It is unlikely that RidA^{HOCl} would universally interact with all *E. coli* 2AA generators or with eukaryotic enzymes, so the effect of RidA^{HOCl} on CdsH or *A. thaliana* IlvA activity might provide alternative platforms to further evaluate chaperone activity *in vitro*. It is possible that HOCl targets Arg105 and might account for decreased deaminase activity of RidA^{HOCl}. This scenario is consistent with the observation that cysteine-free RidA appeared to be chaperone-activated and lacked deaminase activity. Additionally, HOCl is most likely to modify arginine and lysine residues.

A comprehensive model for RidA activity

It is possible that RidA plays a role as a chaperone in addition to its primary function as a 2AA deaminase. Figure 2.5 shows a comprehensive model where unmodified RidA catalyzes 2AA deamination to mitigate metabolic stress. The data that suggest RidA can be chaperone-activated are consistent with a scenario where a modification acts as a switch between two exclusive activities. *In vitro*, the gain of chaperone activity correlated with a loss of 2AA deaminase activity. Similarly, 2AA deaminase activity was compromised by the peroxynitrite modification to Cys107 that led to a redox-sensing role. These observations suggest that a *ridA* mutant strain would face an increased 2AA burden if growing in the presence of either HOCl or peroxynitrite. Therefore, it is likely that activity of several PLP-enzymes would be decreased and that this might ultimately affect growth. The possible proportion of unmodified (2AA deaminase

active) and modified (2AA deaminase deficient) RidA proteins under each condition remains unclear.

2.5 THE RIDA PARADIGM EXTENDS BEYOND BACTERIA

Assimilation of Rid knowledge across fields

In light of recent annotation of the Rid superfamily as enamine/imine deaminases, following elucidation of the 2AA stress response role in *S. enterica*, we reevaluate the collective biochemical data associated with members of this superfamily. With “reference to sequence” online tools, it is becoming easier to identify studies from other fields that pertain to Rid biology. This review addresses publications on RidA and Rid1-7 enzymes from all domains of life, spanning the biological sciences.

S. enterica provided a genetically tractable model system in which the 2AA stress response role of RidA was elucidated through a combination of rigorous genetic and biochemical experiments. Similar experiments have been replicated in plants and yeast, and supported the idea that 2AA stress arises in the absence of a functional RidA protein in these organisms. 2AA deamination is the defining activity of the RidA subfamily and all purified RidA proteins tested to date have catalyzed this reaction. The foundational studies in bacteria urge the investigation of the effects of the reactive 2AA molecule in all domains of life.

Non-RidA proteins

Biochemical surveys and crystal structure analysis of RidA, Rid1 and Rid2 proteins suggested an active site arginine residue was required for deamination of multiple enamine/imine species. A single mutant phenotype has been described in the absence of a non-RidA subfamily protein in *Campylobacter jejuni*. Our understanding of Rid4-7 proteins is represented by two crystal structures, yet no biochemical activities or mutant phenotypes have been described. Since

Rid4-6 proteins lack the critical arginine active site residue, it is unlikely that these enzymes function as deaminases. Complementation studies and purification of *S. enterica* STM1549 (Rid7) seemed to support this hypothesis. In this vein, it is not appropriate to assign the functional role of deaminase to any Rid4-6 proteins at this time.

RidA activity demanded a paradigm shift regarding spontaneous reactions involving water

Elucidation of the RidA mechanism challenged our perspective on cellular water availability and mandated that the differences be explored between cellular chemistry and *in vitro* aqueous chemistry. Work on RidA also refuted the presumption that the 2AA catalytic intermediate remained enzyme-bound and ushered in considerations for the far-reaching effects of reactive metabolites. As we move into studying the more nuanced or stochastic events in metabolic crosstalk, we can probe deeper into metabolite-metabolite interactions. The potential for a Rid enzyme to catalyze a reaction previously attributed to water has been demonstrated with multiple bacterial enzymes (serine/threonine dehydratase, cysteine desulfhydrase, diaminopropionate ammonia-lyase and D-arginine dehydrogenase).

RidA elucidation benefitted from unbiased approaches

A serendipitous mutation was identified within the context of thiamine biosynthesis that eventually led to elucidation of the RidA paradigm. It is likely that solely structure-function approaches would not have been successful at identifying 2-AA as the relevant substrate, given its short half life. Furthermore, development of relevant activity assays was possible only after the identification of PRA accumulation in a *ridA* mutant strain, a phenomenon unrelated to the enzyme's true activity. In hindsight, the accumulation of reactive metabolites is plausible now that the RidA paradigm has been rigorously tested *in vivo*, and reconstituted *in vitro*. However, it is not likely that this scenario would have been envisioned from scratch. Instead simple models

were built upon and refined, providing a strong case for the continued support of curiosity-driven research in the “-omics” age to unravel deeper metabolic connections.

2.6 REFERENCES

1. Niehaus TD, Gerdes S, Hodge-Hanson K, Zhukov A, Cooper AJ, ElBadawi-Sidhu M, et al. Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC genomics*. 2015;16:382.
2. Volz K. A test case for structure-based functional assignment: the 1.2 Å crystal structure of the yjgF gene product from *Escherichia coli*. *Protein science : a publication of the Protein Society*. 1999;8(11):2428-37.
3. Mistiniene E, Luksa V, Sereikaite J, Naktinis V. Oligomeric assembly and ligand binding of the members of protein family YER057c/YIL051c/YJGF. *Bioconjugate chemistry*. 2003;14(6):1243-52.
4. Parsons L, Bonander N, Eisenstein E, Gilson M, Kairys V, Orban J. Solution structure and functional ligand screening of HI0719, a highly conserved protein from bacteria to humans in the YjgF/YER057c/UK114 family. *Biochemistry*. 2003;42(1):80-9.
5. Burman JD, Stevenson CE, Sawers RG, Lawson DM. The crystal structure of *Escherichia coli* TdcF, a member of the highly conserved YjgF/YER057c/UK114 family. *BMC structural biology*. 2007;7:30.
6. Babbitt PC. Definitions of enzyme function for the structural genomics era. *Current opinion in chemical biology*. 2003;7(2):230-7.
7. Schnoes AM, Brown SD, Dodevski I, Babbitt PC. Annotation error in public databases: misannotation of molecular function in enzyme superfamilies. *PLoS Comput Biol*. 2009;5(12):e1000605.
8. Christopherson MR, Schmitz GE, Downs DM. YjgF is required for isoleucine biosynthesis when *Salmonella enterica* is grown on pyruvate medium. *Journal of bacteriology*. 2008;190(8):3057-62.
9. Lambrecht JA, Flynn JM, Downs DM. Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions. *The Journal of biological chemistry*. 2012;287(5):3454-61.
10. Hodge-Hanson KM, Downs DM. 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*. 2017;12(9):e0185544.
11. Lambrecht JA, Schmitz GE, Downs DM. RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life. *mBio*. 2013;4(1):e00033-13.

12. Niehaus TD, Nguyen TN, Gidda SK, ElBadawi-Sidhu M, Lambrecht JA, McCarty DR, et al. Arabidopsis and maize RidA proteins preempt reactive enamine/imine damage to branched-chain amino acid biosynthesis in plastids. *Plant Cell*. 2014;26(7):3010-22.
13. ElRamlawy KG, Fujimura T, Baba K, Kim JW, Kawamoto C, Isobe T, et al. Der f 34, a Novel Major House Dust Mite Allergen Belonging to a Highly Conserved Rid/YjgF/YER057c/UK114 Family of Imine Deaminases. *J Biol Chem*. 2016;291(41):21607-15.
14. Borchert AJ, Downs DM. The Response to 2-Aminoacrylate Differs in *Escherichia coli* and *Salmonella enterica*, despite Shared Metabolic Components. *J Bacteriol*. 2017;199(14).
15. Enos-Berlage JL, Langendorf MJ, Downs DM. Complex metabolic phenotypes caused by a mutation in *yjgF*, encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of bacteriology*. 1998;180(24):6519-28.
16. Browne BA, Ramos AI, Downs DM. PurF-independent phosphoribosyl amine formation in *yjgF* mutants of *Salmonella enterica* utilizes the tryptophan biosynthetic enzyme complex anthranilate synthase-phosphoribosyltransferase. *J Bacteriol*. 2006;188(19):6786-92.
17. Lambrecht JA, Browne BA, Downs DM. Members of the YjgF/YER057c/UK114 family of proteins inhibit phosphoribosylamine synthesis in vitro. *J Biol Chem*. 2010;285(45):34401-7.
18. Christopherson MR, Lambrecht JA, Downs D, Downs DM. Suppressor analyses identify threonine as a modulator of *ridA* mutant phenotypes in *Salmonella enterica*. *PloS one*. 2012;7(8):e43082.
19. Eliot AC, Kirsch JF. Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. *Annual review of biochemistry*. 2004;73:383-415.
20. Schnackerz KD, Ehrlich JH, Gieseemann W, Reed TA. Mechanism of action of D-serine dehydratase. Identification of a transient intermediate. *Biochemistry*. 1979;18(16):3557-63.
21. Datta P, Bhadra R. Biodegradative threonine dehydratase. Reduction of ferricyanide by an intermediate of the enzyme-catalyzed reaction. *European journal of biochemistry*. 1978;91(2):527-32.
22. Phillips AT, Wood WA. The mechanism of action of 5'-adenylic acid-activated threonine dehydrase. *The Journal of biological chemistry*. 1965;240(12):4703-9.
23. Chargaff E, Sprinson DB. Studies on the mechanism of deamination of serine and threonine in biological systems. *Journal of Biological Chemistry*. 1943;151(1):273-80.

24. Kredich NM, Foote LJ, Keenan BS. The stoichiometry and kinetics of the inducible cysteine desulphydrase from *Salmonella typhimurium*. *The Journal of biological chemistry*. 1973;248(17):6187-96.
25. Collins JM, Monty KJ. The cysteine desulphydrase of *Salmonella typhimurium*. Kinetic and catalytic properties. *The Journal of biological chemistry*. 1973;248(17):5943-9.
26. Phillips RS, Demidkina TV, Faleev NG. Structure and mechanism of tryptophan indole-lyase and tyrosine phenol-lyase. *Biochimica et biophysica acta*. 2003;1647(1-2):167-72.
27. Demidkina TV, Anston AA, Faleev NG, Phillips RS, Zakomyrdina LN. Spatial structure and mechanism of tyrosine phenol-lyase and tryptophan indole-lyase. *Molekuliarnaia biologii*. 2009;43(2):295-308.
28. Barends TR, Domratcheva T, Kulik V, Blumenstein L, Niks D, Dunn MF, et al. Structure and mechanistic implications of a tryptophan synthase quinonoid intermediate. *Chembiochem : a European journal of chemical biology*. 2008;9(7):1024-8.
29. Ernst DC, Lambrecht JA, Schomer RA, Downs DM. Endogenous synthesis of 2-aminoacrylate contributes to cysteine sensitivity in *Salmonella enterica*. *Journal of bacteriology*. 2014;196(18):3335-42.
30. Ernst DC, Anderson ME, Downs DM. L-2,3-diaminopropionate generates diverse metabolic stresses in *Salmonella enterica*. *Mol Microbiol*. 2016;101(2):210-23.
31. Downs DM, Ernst DC. From microbiology to cancer biology: the Rid protein family prevents cellular damage caused by endogenously generated reactive nitrogen species. *Molecular microbiology*. 2015;96(2):211-9.
32. Schmitz G, Downs DM. 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. *Journal of bacteriology*. 2004;186(3):803-10.
33. Flynn JM, Downs DM. In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5'-phosphate cofactor. *Journal of bacteriology*. 2013;195(16):3603-9.
34. Flynn JM, Christopherson MR, Downs DM. Decreased coenzyme A levels in ridA mutant strains of *Salmonella enterica* result from inactivated serine hydroxymethyltransferase. *Molecular microbiology*. 2013;89(4):751-9.
35. Kim JM, Yoshikawa H, Shirahige K. A member of the YER057c/yjgF/Uk114 family links isoleucine biosynthesis and intact mitochondria maintenance in *Saccharomyces cerevisiae*. *Genes to cells : devoted to molecular & cellular mechanisms*. 2001;6(6):507-17.
36. Reid AN, Pandey R, Palyada K, Whitworth L, Doukhanine E, Stintzi A. Identification of *Campylobacter jejuni* genes contributing to acid adaptation by transcriptional profiling

- and genome-wide mutagenesis. *Applied and environmental microbiology*. 2008;74(5):1598-612.
37. Flint A, Sun YQ, Butcher J, Stahl M, Huang H, Stintzi A. Phenotypic screening of a targeted mutant library reveals *Campylobacter jejuni* defenses against oxidative stress. *Infect Immun*. 2014;82(6):2266-75.
 38. Reuter M, Periago PM, Mulholland F, Brown HL, van Vliet AH. A PAS domain-containing regulator controls flagella-flagella interactions in *Campylobacter jejuni*. *Front Microbiol*. 2015;6:770.
 39. Lambrecht JA, Downs DM. Anthranilate phosphoribosyl transferase (TrpD) generates phosphoribosylamine for thiamine synthesis from enamines and phosphoribosyl pyrophosphate. *ACS Chem Biol*. 2013;8(1):242-8.
 40. Kreuzer-Martin HW, Lott MJ, Ehleringer JR, Hegg EL. Metabolic processes account for the majority of the intracellular water in log-phase *Escherichia coli* cells as revealed by hydrogen isotopes. *Biochemistry*. 2006;45(45):13622-30.
 41. Zhou HX, Rivas G, Minton AP. Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu Rev Biophys*. 2008;37:375-97.
 42. Mistiniene E, Pozdniakovaite N, Pependikyte V, Naktinis V. Structure-based ligand binding sites of protein p14.5, a member of protein family YER057c/YIL051c/YjgF. *International journal of biological macromolecules*. 2005;37(1-2):61-8.
 43. Oka T, Tsuji H, Noda C, Sakai K, Hong YM, Suzuki I, et al. Isolation and characterization of a novel perchloric acid-soluble protein inhibiting cell-free protein synthesis. *The Journal of biological chemistry*. 1995;270(50):30060-7.
 44. Morishita R, Kawagoshi A, Sawasaki T, Madin K, Ogasawara T, Oka T, et al. Ribonuclease activity of rat liver perchloric acid-soluble protein, a potent inhibitor of protein synthesis. *The Journal of biological chemistry*. 1999;274(29):20688-92.
 45. Schmiedeknecht G, Kerkhoff C, Orso E, Stohr J, Aslanidis C, Nagy GM, et al. Isolation and characterization of a 14.5-kDa trichloroacetic-acid-soluble translational inhibitor protein from human monocytes that is upregulated upon cellular differentiation. *European journal of biochemistry*. 1996;242(2):339-51.
 46. Dhawan L, Liu B, Pytlak A, Kulshrestha S, Blaxall BC, Taubman MB. Y-box binding protein 1 and RNase UK114 mediate monocyte chemoattractant protein 1 mRNA stability in vascular smooth muscle cells. *Molecular and cellular biology*. 2012;32(18):3768-75.
 47. Su P, Feng T, Zhou X, Zhang S, Zhang Y, Cheng J, et al. Isolation of Rhp-PSP, a member of YER057c/YjgF/UK114 protein family with antiviral properties, from the

- photosynthetic bacterium *Rhodospseudomonas palustris* strain JSC-3b. *Scientific reports*. 2015;5:16121.
48. Matsumoto M, Kanouchi H, Suzuki K, Kaneki K, Kawasaki Y, Oka T. Purification and characterization of perchloric acid soluble protein from rat lung. *Comp Biochem Physiol B Biochem Mol Biol*. 2003;135(2):255-62.
 49. Kanouchi H, Miyamoto M, Oka T, Matsumoto M, Okamoto T, Tone S, et al. Perchloric acid-soluble protein is expressed in enterocytes and goblet cells in the intestine and upregulated by dietary lipid. *Biochimica et biophysica acta*. 2006;1760(9):1380-5.
 50. Antonenkov VD, Ohlmeier S, Sormunen RT, Hiltunen JK. UK114, a YjgF/Yer057p/UK114 family protein highly conserved from bacteria to mammals, is localized in rat liver peroxisomes. *Biochemical and biophysical research communications*. 2007;357(1):252-7.
 51. Samuel SJ, Tzung SP, Cohen SA. Hrp12, a novel heat-responsive, tissue-specific, phosphorylated protein isolated from mouse liver. *Hepatology*. 1997;25(5):1213-22.
 52. Pozdniakovaite N, Pependikyte V. DNA methylation differences in human p14.5 gene promoter region in normal and proliferating cells. *Development, growth & differentiation*. 2005;47(7):493-9.
 53. Kanouchi H, Matsumoto M, Taga M, Yamada K, Oka T, Tone S, et al. Nuclear transfer of perchloric acid-soluble protein by endoplasmic reticulum stressors. *Protein science : a publication of the Protein Society*. 2005;14(9):2344-9.
 54. D'Inca R, Marteil G, Bazile F, Pascal A, Guitton N, Lavigne R, et al. Proteomic screen for potential regulators of M-phase entry and quality of meiotic resumption in *Xenopus laevis* oocytes. *Journal of proteomics*. 2010;73(8):1542-50.
 55. Leitner-Dagan Y, Ovadis M, Zuker A, Shklarman E, Ohad I, Tzfira T, et al. CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. *Planta*. 2006;225(1):89-102.
 56. Oxelmark E, Marchini A, Malanchi I, Magherini F, Jaquet L, Hajibagheri MA, et al. Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome. *Molecular and cellular biology*. 2000;20(20):7784-97.
 57. Rain JC, Selig L, De Reuse H, Battaglia V, Reverdy C, Simon S, et al. The protein-protein interaction map of *Helicobacter pylori*. *Nature*. 2001;409(6817):211-5.
 58. Lopez-Campistrous A, Semchuk P, Burke L, Palmer-Stone T, Brokx SJ, Broderick G, et al. Localization, annotation, and comparison of the *Escherichia coli* K-12 proteome under two states of growth. *Mol Cell Proteomics*. 2005;4(8):1205-9.

59. de Vries SP, Eleveld MJ, Hermans PW, Bootsma HJ. Characterization of the molecular interplay between *Moraxella catarrhalis* and human respiratory tract epithelial cells. *PloS one*. 2013;8(8):e72193.
60. Ceciliani F, Faotto L, Negri A, Colombo I, Berra B, Bartorelli A, et al. The primary structure of UK114 tumor antigen. *FEBS Letters*. 1996;393:147-50.
61. Bussolati G, Geuna M, Bussolati B, Millesimo M, Botta M, Bartorelli A. Cytolytic and tumor inhibitory antibodies against UK114 protein in the sera of cancer patients. *International Journal of Oncology*. 1997;10(1019-6439 (Print)):779-85.
62. Chong CL, Huang SF, Hu CP, Chen YL, Chou HY, Chau GY, et al. Decreased expression of UK114 is related to the differentiation status of human hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2008;17(3):535-42.
63. Kaneki K, Kanouchi H, Matsumoto M, Kawasaki Y, Akuzawa M, Oka T. Down regulation of a novel protein, PSP, in rat hepatoma cdRLh 84-beared tumor. *The Journal of veterinary medical science*. 2003;65(7):781-5.
64. Kanouchi H, Taga M, Okamoto T, Yamasaki M, Oka T, Yamada K, et al. Reduced expression of perchloric acid-soluble protein after partial hepatectomy in rats. *Biosci Biotechnol Biochem*. 2006;70(1):290-2.
65. Levy-Favatier F, Leroux A, Antoine B, Nedelec B, Delpech M. Upregulation of rat P23 (a member of the YjgF protein family) by fasting, glucose diet and fatty acid feeding. *Cellular and molecular life sciences : CMLS*. 2004;61(22):2886-92.
66. Emery SJ, Mirzaei M, Vuong D, Pascovici D, Chick JM, Lacey E, et al. Induction of virulence factors in *Giardia duodenalis* independent of host attachment. *Scientific reports*. 2016;6:20765.
67. Beloin C, Valle J, Latour-Lambert P, Faure P, Kzreminski M, Balestrino D, et al. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Molecular microbiology*. 2004;51(3):659-74.
68. Mangalappalli-Illathu AK, Vidovic S, Korber DR. Differential adaptive response and survival of *Salmonella enterica* serovar enteritidis planktonic and biofilm cells exposed to benzalkonium chloride. *Antimicrobial agents and chemotherapy*. 2008;52(10):3669-80.
69. Ternan NG, Jain S, Srivastava M, McMullan G. Comparative transcriptional analysis of clinically relevant heat stress response in *Clostridium difficile* strain 630. *PloS one*. 2012;7(7):e42410.
70. Boulette ML, Baynham PJ, Jorth PA, Kukavica-Ibrulj I, Longoria A, Barrera K, et al. Characterization of alanine catabolism in *Pseudomonas aeruginosa* and its importance for proliferation in vivo. *Journal of bacteriology*. 2009;191(20):6329-34.

71. LaSarre B, Federle MJ. Regulation and consequence of serine catabolism in *Streptococcus pyogenes*. *Journal of bacteriology*. 2011;193(8):2002-12.
72. Chou HT, Kwon DH, Hegazy M, Lu CD. Transcriptome analysis of agmatine and putrescine catabolism in *Pseudomonas aeruginosa* PAO1. *Journal of bacteriology*. 2008;190(6):1966-75.
73. Liu M, Hanks TS, Zhang J, McClure MJ, Siemsen DW, Elser JL, et al. Defects in ex vivo and in vivo growth and sensitivity to osmotic stress of group A *Streptococcus* caused by interruption of response regulator gene *vicR*. *Microbiology*. 2006;152(Pt 4):967-78.
74. Borchert AJ, Downs DM. Endogenously generated 2-aminoacrylate inhibits motility in *Salmonella enterica*. *Scientific reports*. 2017;7(1):12971.
75. Zelyas NJ, Cai H, Kwong T, Jensen SE. Alanylclavam biosynthetic genes are clustered together with one group of clavulanic acid biosynthetic genes in *Streptomyces clavuligerus*. *Journal of bacteriology*. 2008;190(24):7957-65.
76. He Z, Spain JC. A novel 2-aminomuconate deaminase in the nitrobenzene degradation pathway of *Pseudomonas pseudoalcaligenes* JS45. *Journal of bacteriology*. 1998;180(9):2502-6.
77. Davis JK, He Z, Somerville CC, Spain JC. Genetic and biochemical comparison of 2-aminophenol 1,6-dioxygenase of *Pseudomonas pseudoalcaligenes* JS45 to meta-cleavage dioxygenases: divergent evolution of 2-aminophenol meta-cleavage pathway. *Archives of microbiology*. 1999;172(5):330-9.
78. Takenaka S, Murakami S, Kim YJ, Aoki K. Complete nucleotide sequence and functional analysis of the genes for 2-aminophenol metabolism from *Pseudomonas* sp. AP-3. *Archives of microbiology*. 2000;174(4):265-72.
79. Muraki T, Taki M, Hasegawa Y, Iwaki H, Lau PC. Prokaryotic homologs of the eukaryotic 3-hydroxyanthranilate 3,4-dioxygenase and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase in the 2-nitrobenzoate degradation pathway of *Pseudomonas fluorescens* strain KU-7. *Applied and environmental microbiology*. 2003;69(3):1564-72.
80. Oori C, Takenaka S, Murakami S, Aoki K. A novel coupled enzyme assay reveals an enzyme responsible for the deamination of a chemically unstable intermediate in the metabolic pathway of 4-amino-3-hydroxybenzoic acid in *Bordetella* sp. strain 10d. *European journal of biochemistry*. 2004;271(15):3248-54.
81. Zhou L, Wang JY, Wu J, Wang J, Poplawsky A, Lin S, et al. The diffusible factor synthase XanB2 is a bifunctional chorismatase that links the shikimate pathway to ubiquinone and xanthomonadins biosynthetic pathways. *Molecular microbiology*. 2013;87(1):80-93.

82. Link AJ, Robison K, Church GM. Comparing the predicted and observed properties of proteins encoded in the genome of *Escherichia coli* K-12. *Electrophoresis*. 1997;18(8):1259-313.
83. Wasinger VC, Humphery-Smith I. Small genes/gene-products in *Escherichia coli* K-12. *FEMS microbiology letters*. 1998;169(2):375-82.
84. Rangan KJ, Yang YY, Charron G, Hang HC. Rapid visualization and large-scale profiling of bacterial lipoproteins with chemical reporters. *Journal of the American Chemical Society*. 2010;132(31):10628-9.
85. Lindemann C, Lupilova N, Muller A, Warscheid B, Meyer HE, Kuhlmann K, et al. Redox proteomics uncovers peroxynitrite-sensitive proteins that help *Escherichia coli* to overcome nitrosative stress. *The Journal of biological chemistry*. 2013;288(27):19698-714.
86. Melloni E, Michetti M, Salamino F, Pontremoli S. Molecular and functional properties of a calpain activator protein specific for mu-isoforms. *The Journal of biological chemistry*. 1998;273(21):12827-31.
87. Farkas A, Nardai G, Csermely P, Tompa P, Friedrich P. DUK114, the *Drosophila* orthologue of bovine brain calpain activator protein, is a molecular chaperone. *The Biochemical journal*. 2004;383(Pt 1):165-70.
88. Levy-Favatier F, Cuisset L, Nedelec B, Tichonicky L, Kruh J, Delpech M. Characterization, purification and cDNA cloning of a rat perchloric-acid-soluble 23-kDa protein present only in liver and kidney. *European journal of biochemistry*. 1993;212(3):665-73.
89. Enos-Berlage JLL, Mark J.; Downs, Diana M. Complex metabolic phenotypes caused by a mutation in *yjgF* encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of Bacteriology*. 1998;180(24):6519-28.
90. Oxelmark EM, Antonio; Malanchi, Ilaria; Magherini, Francesca; Jaquet, Laurence; Hajibagheri, M. A. Nasser; Blight, Kenneth J.; Jauniaux, Jean-Claude; Tommassino, Massimo. Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome. *Molecular and cellular biology*. 2000;20(20):7784-97.
91. Muller A, Langklotz S, Lupilova N, Kuhlmann K, Bandow JE, Leichert LI. Activation of RidA chaperone function by N-chlorination. *Nature communications*. 2014;5:5804.
92. Dahl JU, Gray MJ, Jakob U. Protein quality control under oxidative stress conditions. *J Mol Biol*. 2015;427(7):1549-63.
93. Voth W, Jakob U. Stress-Activated Chaperones: A First Line of Defense. *Trends in biochemical sciences*. 2017.

94. Kim S, Jeong H, Kim EY, Kim JF, Lee SY, Yoon SH. Genomic and transcriptomic landscape of *Escherichia coli* BL21(DE3). *Nucleic Acids Res.* 2017;45(9):5285-93.
95. Zhang HM, Gao Y, Li M, Chang WR. Crystal structure of the PSPTO-PSP protein from *Pseudomonas syringae* pv. tomato str. DC3000 in complex with D-glucose. *Biochemical and biophysical research communications.* 2010;397(1):82-6.
96. Sinha S, Rappu P, Lange SC, Mantsala P, Zalkin H, Smith JL. Crystal structure of *Bacillus subtilis* YabJ, a purine regulatory protein and member of the highly conserved YjgF family. *Proc Natl Acad Sci U S A.* 1999;96(23):13074-9.
97. Deaconescu AM, Roll-Mecak A, Bonanno JB, Gerchman SE, Kycia H, Studier FW, et al. X-ray structure of *Saccharomyces cerevisiae* homologous mitochondrial matrix factor 1 (Hmf1). *Proteins.* 2002;48(2):431-6.
98. Deriu D, Briand C, Mistiniene E, Naktinis V, Grutter MG. Structure and oligomeric state of the mammalian tumour-associated antigen UK114. *Acta crystallographica Section D, Biological crystallography.* 2003;59(Pt 9):1676-8.
99. Manjasetty BA, Delbruck H, Pham DT, Mueller U, Fieber-Erdmann M, Scheich C, et al. Crystal structure of *Homo sapiens* protein hp14.5. *Proteins.* 2004;54(4):797-800.
100. Miyakawa T, Lee WC, Hatano K, Kato Y, Sawano Y, Miyazono K, et al. Crystal structure of the YjgF/YER057c/UK114 family protein from the hyperthermophilic archaeon *Sulfolobus tokodaii* strain 7. *Proteins.* 2006;62(2):557-61.
101. Liu X, Zeng J, Chen X, Xie W. Crystal structures of RidA, an important enzyme for the prevention of toxic side products. *Scientific reports.* 2016;6:30494.
102. Pu YG, Jiang YL, Ye XD, Ma XX, Guo PC, Lian FM, et al. Crystal structures and putative interface of *Saccharomyces cerevisiae* mitochondrial matrix proteins Mmf1 and Mam33. *J Struct Biol.* 2011;175(3):469-74.
103. Thakur KG, Praveena T, Gopal B. *Mycobacterium tuberculosis* Rv2704 is a member of the YjgF/YER057c/UK114 family. *Proteins.* 2010;78(3):773-8.
104. Knapik AA, Petkowski JJ, Otwinowski Z, Cymborowski MT, Cooper DR, Chruszcz M, et al. Structure of *Escherichia coli* RutC, a member of the YjgF family and putative aminoacrylate peracid reductase of the rut operon. *Acta crystallographica Section F, Structural biology and crystallization communications.* 2012;68(Pt 11):1294-9.
105. Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M, et al. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic acids research.* 2005;33(17):5691-702.

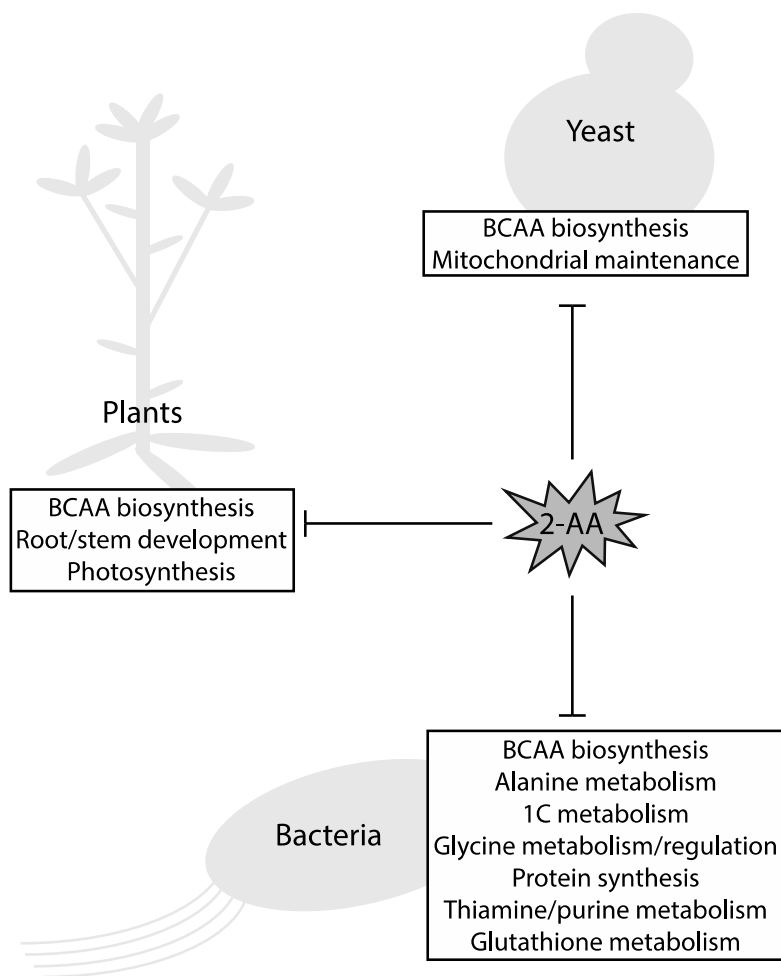


Figure 2.1 Mutant phenotypes indicate pathways affected in the absence of RidA

Mutant phenotypes have been reported for organisms that lack a functional RidA homolog from bacteria (*S. enterica* (8, 15, 16, 18, 29, 30, 33, 34), *E. coli* (14) and *P. aeruginosa* (Hodge-Hanson, unpublished data), plants (*A. thaliana* (1) and *L. esculentum* (55), and the yeast *S. cerevisiae* (35, 56). This model implicates 2-aminoacrylate (2AA) as the cause of downstream effects that affect multiple areas of metabolism.

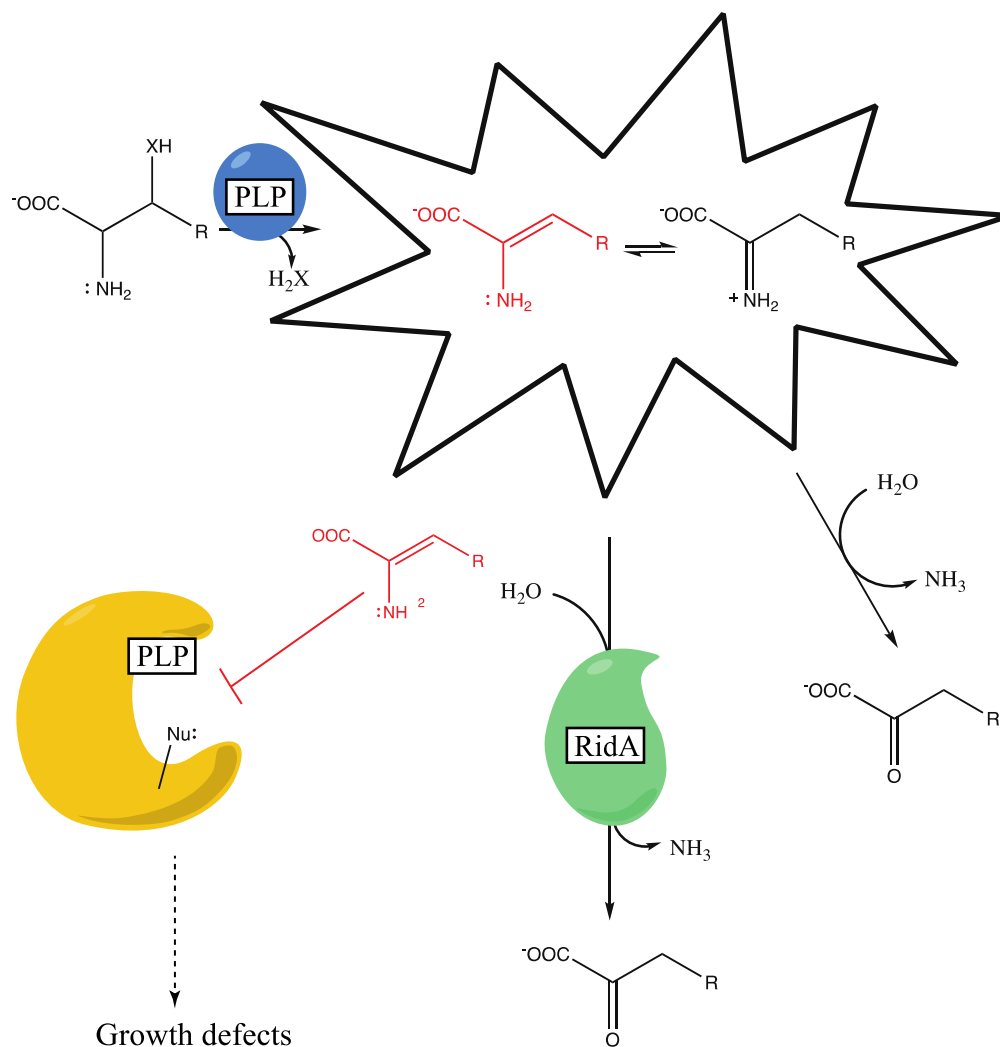


Figure 2.2 The RidA paradigm

The activities of various PLP-dependent enzymes (in blue) can lead to accumulation of the enamine 2-aminoacrylate (2AA, in red), which may tautomerize to the imine species. These intermediates have one of three fates: i) 2AA diffuses throughout the cell and inactivates certain PLP-dependent enzymes (in yellow) ii) the intermediates are deaminated by a Rid protein such as RidA (in green) or iii) the intermediates are non-enzymatically hydrolyzed by solvent water. Inactivation of PLP-enzymes through mechanism (i) can collectively lead to growth defects while mechanisms (ii) and (iii) result in formation of a ketoacid that is less reactive than either intermediate species.

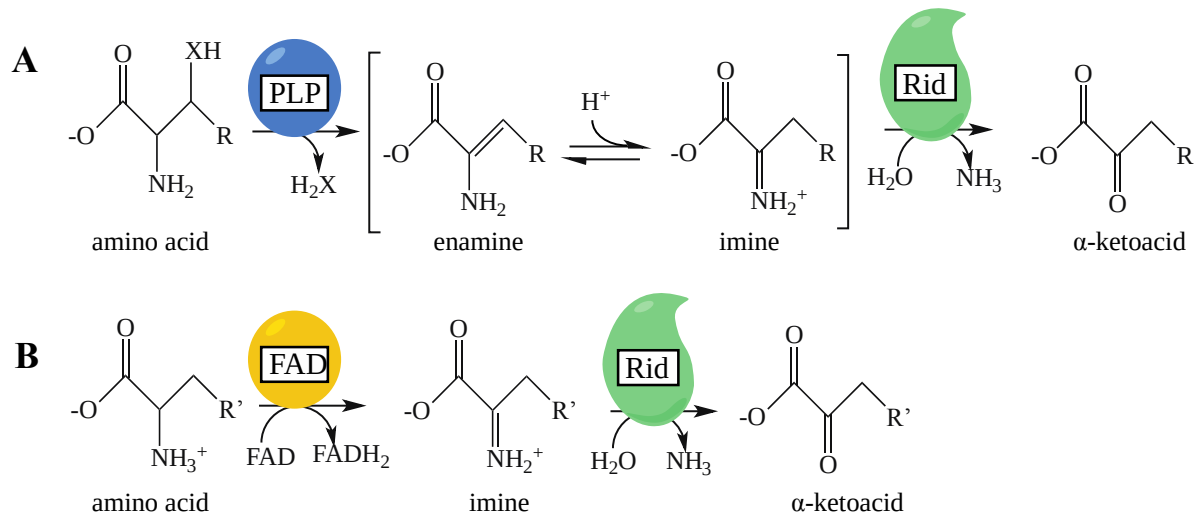


Figure 2.3 PLP- and FAD-dependent mechanisms of imine production

(A) The PLP-dependent generation of a enamine/imine intermediates (shown in brackets) from an amino acid. The intermediates are deaminated by solvent water or by Rid proteins which produces an α -ketoacid. The representations are: threonine, $R = -CH_3$, $X = O$; serine $R = -H$, $X = O$, cysteine $R = -H$, $X = S$. **(B)** The FAD-dependent imine production from an amino acid. Imines are deaminated by solvent water or the reaction is facilitated by Rid proteins. The amino acid functional group in the diagram is represented by R' .

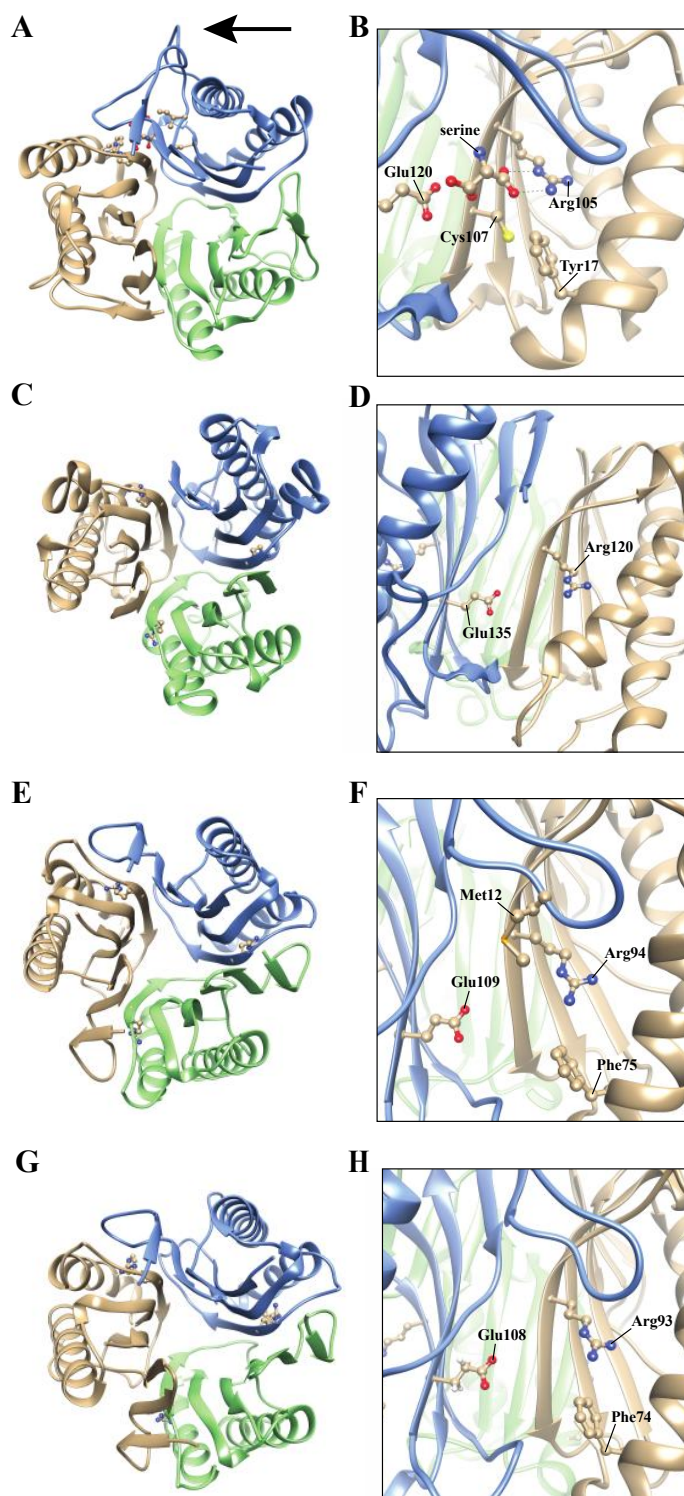


Figure 2.4 Rid structures

RidA homolog (TdcF) from *Escherichia coli* (**A**) homotrimer and (**B**) active site with serine bound. A dotted line is shown between the arginine guanidinium moiety and the serine carboxyl group (2.8Å). An arrow indicates the location of the substrate binding loop (5). The homotrimeric structure and active sites are shown for PA0814 (Rid1) (**C**, **D**), PSPTO_0102 (Rid2) (**E**, **F**), and PA5083 (**G**, **H**).

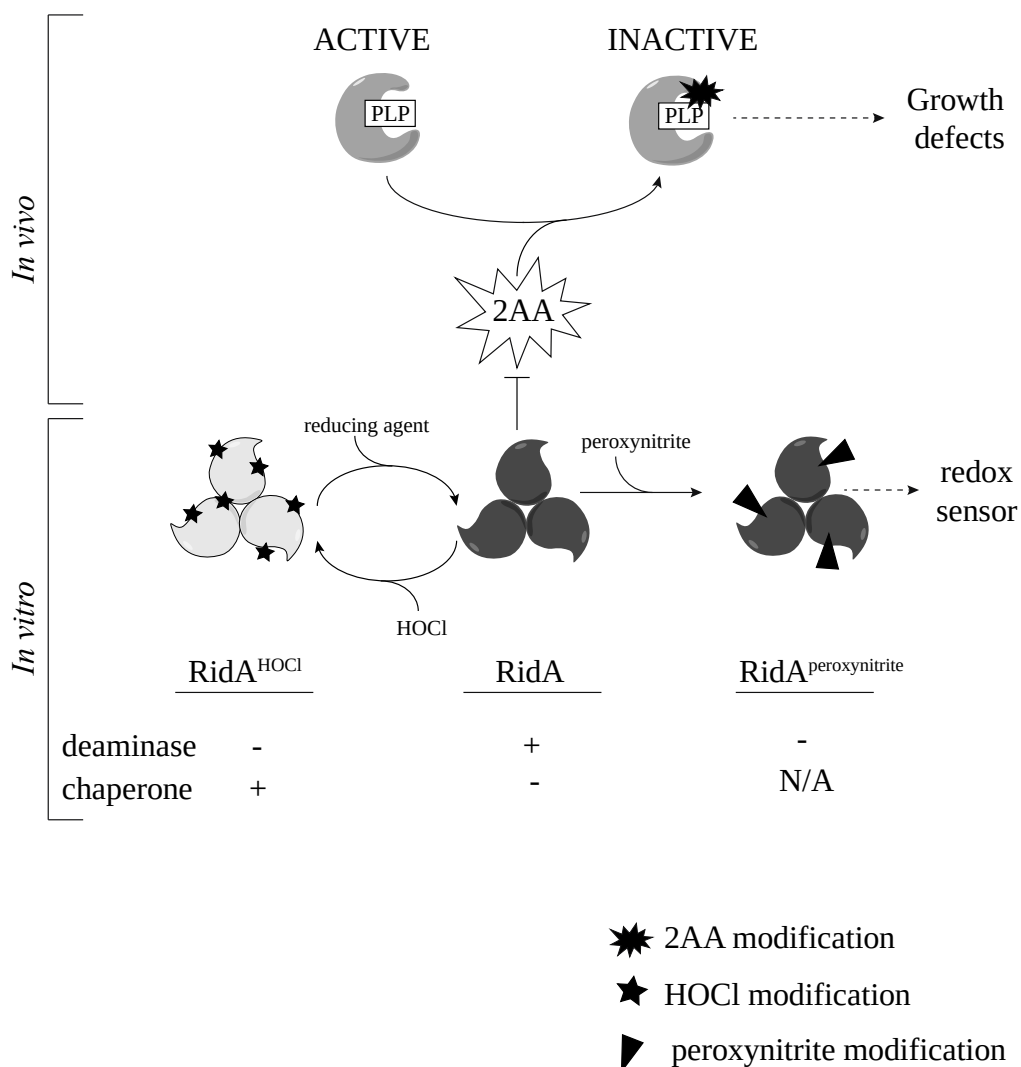


Figure 2.5 A model for wild type and modified RidA proteins

RidA proteins from all domains of life prevent damage to various PLP-enzymes via 2AA deaminase activity (11). Modification by peroxynitrite is attributed to a gain of function as a redox sensor in *E. coli* MG1655 (85). Modification of *E. coli* RidA by HOCl or MCA introduced chaperone activity *in vitro* (91). 2AA deaminase activity decreased by peroxynitrite modification of *E. coli* RidA Cys107 and following HOCl treatment. The exact location(s) of HOCl or MCA modifications remain unknown and are indicated arbitrarily in the figure. Abbreviations are: PLP (pyridoxal 5'-phosphate), 2AA (2-aminoacrylate), HOCl (hypochlorite), MCA (monochloramine).

Table 2.1 Rid structures

Rid subfamily	Protein	Gene name/locus tag	Organism	PDB	Ligand	Reference	Deposited
RidA	PSPTO-PSP	PSPTO_0072	<i>Psuedomonas sryingae</i>	3K0T	D-glucose	(95)	2009
	RidA/YjgF	<i>ridA/yjgF</i>	<i>Escherichia coli</i>	1QU9		(2)	1999
	YabJ	<i>yabJ</i>	<i>Bacillus subtilis</i>	1QD9		(96)	1999
	Hmf1	YER057c	<i>Saccharomyces cerevisiae</i>	1JD1		(97)	2001
	L-PSP	Hrsp12	<i>Rattus norvegicus</i> (rat)	1QAH		to be published	1999
	UK114	Hrsp12	<i>Capra hircus</i> (goat)	1NQ3		(98)	2003
	Hp14.5	HRSP12	<i>Homo sapiens</i> (human)	1ONI	benzoate	(99)	2003
	TdcF	<i>tdcF</i>	<i>Escherichia coli</i>	2UYJ	ethylene glycol	(5)	2007
	TdcF	<i>tdcF</i>	<i>Escherichia coli</i>	2UYP	propionate	(5)	2007
	TdcF	<i>tdcF</i>	<i>Escherichia coli</i>	2UYK	serine	(5)	2007
	TdcF	<i>tdcF</i>	<i>Escherichia coli</i>	2UYN	2-ketobutyrate	(5)	2007
	ST0811	STK_08110	<i>Sulfolobus tokodaii</i>	1X25		(100)	2005
	HI0719	<i>HI_0719</i>	<i>Haemophilus influenzae</i>	1J7H		(4)	2001
	RidA	RIDA	<i>Arabidopsis thaliana</i>	5HP7	apo form	(101)	2016
	RidA	RIDA	<i>Arabidopsis thaliana</i>	5HP8	pyruvate	(101)	2016
	SPy2060	Spy_2060	<i>Streptococcus pyogenes</i>	2EWC	glycerol	to be published	2005
	Cth_2968	Cthe_2798	<i>Clostridium thermocellum</i>	1XRG		to be published	2004
	PA3499	PA3499	<i>Pseudomonas aeruginosa</i>	2IG8		to be published	2006
	TTHA0137	TTHA0137	<i>Thermus thermophilus</i>	2CVL		to be published	2005
	TTHA0137	TTHA0137	<i>Thermus thermophilus</i>	2CW4		to be published	2005
	APE1501	APE1501	<i>Aeropyrum pernix</i>	2CWJ		to be published	2005
	Mmf1	Mmf1	<i>Saccharomyces cerevisiae</i>	3QUW		(102)	2011
	TM0215	TM0215	<i>Thermotoga maritima</i>	2B33		to be published	2005
	PH0854	PH0854	<i>Pyrococcus horikoshii</i>	2DYY		to be published	2006
Rid1	PA0814	PA0814	<i>Pseudomonas aeruginosa</i>				TBD
Rid2	YoaB	<i>yoaB</i>	<i>Salmonella typhimurium</i>	3GTZ	glycerol	to be published	2009
	NMB1025	NMB1025	<i>Neisseria meningitidis</i>	3KJJ	glycerol	to be published	2009
	NMB1025	NMB1025	<i>Neisseria meningitidis</i>	3KJK		to be published	2009
	PSPTO_0102	PSPTO_0102	<i>Psuedomonas sryingae</i>				TBD
	PA5083	PA5083	<i>Pseudomonas aeruginosa</i>				TBD
Rid6	Rv2704	MT2777.1	<i>Mycobacterium tuberculosis</i>	3I7T		(103)	2009
Rid7	YjgH	<i>yjgH</i>	<i>Escherichia coli</i>	1PF5		to be published	2003
RutC	RutC	<i>rutC</i>	<i>Escherichia coli</i>	3V4D		(104)	2011

Table 2.2 Distribution of Rid proteins in representative organisms

Organism	Rid Proteins Encoded ^a								
	Total	RidA	Rid1	Rid2	Rid3	Rid4	Rid5	Rid6	Rid7
Archaea									
<i>Pyrococcus furiosus</i>	1	1							
<i>Sulfolobus tokodaii</i>	1	1							
Bacteria									
Actinobacteria									
<i>Mycobacterium smegmatis</i>	9	4	4			1			
<i>Mycobacterium tuberculosis</i>	2		1					1	
<i>Streptomyces coelicolor</i>	12	9	1					2	
<i>Streptomyces griseus</i>	8	5	1			1		1	
Bacteroidetes									
<i>Bacteroides fragilis</i>	2	1					1		
<i>Deinococcus-Thermus</i>									
<i>Thermus thermophilus</i>	1	1							
Firmicutes									
<i>Clostridium difficile</i>	3	2		1					
<i>Bacillus subtilis</i>	1	1							
<i>Streptococcus pyogenes</i>	2	2							
Alphaproteobacteria									
<i>Bradyrhizobium japonicum</i>	16	9	1	5				1	
<i>Caulobacter crescentus</i> ^b	3	1	1		1				
<i>Rhodopseudomonas palustris</i>	6	3	1					1	1
Betaproteobacteria									
<i>Neisseria meningitidis</i>	2	1		1					
<i>Bordetella pertussis</i> CS	9	6	1	1					1
<i>Burkholderia cepacia</i>	17	8	3	2			1	1	2
Deltaproteobacteria									
<i>Campylobacter jejuni</i>	2	1		1					
<i>Myxococcus xanthus</i>	4	4							
<i>Helicobacter pylori</i>	1	1							
Gammaproteobacteria									
<i>Acinetobacter baylyi</i> ^b	7	4	1	2					
<i>Erwinia amylovora</i>	2			1				1	
<i>Escherichia coli</i> ^b	4	2		1					1
<i>Haemophilus influenzae</i>	3	2		1					
<i>Pseudomonas aeruginosa</i>	9	4	1	3					1
<i>Pseudomonas putida</i>	10	7		2			1		
<i>Pseudomonas syringae</i> ^b	8	5		1	1				1
<i>Salmonella enterica</i>	3	1		1					1
<i>Yersinia pestis</i>	3	3							
Eukarya									
<i>Saccharomyces cerevisiae</i>	2	2							
<i>Arabidopsis thaliana</i>	2	2							
<i>Zea mays</i>	1	1							
<i>Homo sapiens</i>	1	1							

^a Genomes and annotations accessed from the PubSEED database (105).

^b Organisms additionally encodes RutC, a member of the Rid superfamily that has not been assigned a subfamily.

Table 2.3 Validated Rid deaminases

Organism	Protein	Subfamily	Reference
Archaea			
<i>Pyrococcus furiosus</i>	PF0668	RidA	(11)
Bacteria			
<i>Salmonella enterica</i>	RidA	RidA	(9)
<i>Salmonella enterica</i>	YoaB	Rid2	(1)
<i>Escherichia coli</i>	RidA	RidA	(14)
<i>Escherichia coli</i>	TdcF	RidA	(14)
<i>Escherichia coli</i>	RutC	N/A ^a	(9)
<i>Bacillus subtilis</i>	YabJ	RidA	(9)
<i>Acinetobacter baylyi</i>	ACIAD3089	Rid1	(10)
<i>Pseudomonas aeruginosa</i>	PA0814	Rid1	(10)
<i>Pseudomonas aeruginosa</i>	PA5083	Rid2	(10)
<i>Pseudomonas syringae</i>	PSPTO_0102	Rid2	(10)
<i>Pseudomonas syringae</i>	PSPTO_3006	Rid3	(10)
<i>Pseudomonas fluorescens</i>	PFL_1385	Rid3	(10)
Eukarya			
<i>Homo sapiens</i>	UK114	RidA	(11)
<i>Cucumis sativus</i>	ChrD	RidA	(11)
<i>Arabidopsis thaliana</i>	RidA	RidA	(12, 101)
<i>Zea mays</i>	RidA	RidA	(12)
<i>Dermatophagoides farinae</i> (house dust mite)	Derf34	RidA	(13)
<i>Saccharomyces cerevisiae</i>	Hmf1	RidA	(Ernst, 2017, submitted)

^a RutC has not been assigned to a subfamily.

CHAPTER 3

MEMBERS OF THE RID PROTEIN FAMILY HAVE BROAD IMINE DEAMINASE
ACTIVITY AND CAN ACCELERATE THE *PSEUDOMONAS AERUGINOSA* D-ARGININE
DEHYDROGENASE (DAUA) REACTION *IN VITRO*²

² Hodge-Hanson, K. M., D. M. Downs. 2017. *PLoS One* 12(9) e:0185544.
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3.1 ABSTRACT

The Rid (YjgF/YER057c/UK114) protein family is a group of small, sequence diverse proteins that consists of eight subfamilies. The archetypal RidA subfamily is found in all domains, while the Rid1-7 subfamilies are present only in prokaryotes. Bacterial genomes often encode multiple members of the Rid superfamily. The best characterized member of this protein family, RidA from *Salmonella enterica*, is a deaminase that quenches the reactive metabolite 2-aminoacrylate generated by pyridoxal 5'-phosphate-dependent enzymes and ultimately spares certain enzymes from damage. The accumulation of 2-aminoacrylate can damage enzymes and lead to growth defects in bacteria, plants, and yeast. While all subfamily members have been annotated as imine deaminases based on the RidA characterization, experimental evidence to support this annotation exists for a single protein outside the RidA subfamily. Our understanding of the remaining subfamilies is limited. Here we report that six proteins, spanning Rid subfamilies 1-3, deaminate a variety of imine/enamine substrates with differing specific activities. Proteins from the Rid2 and Rid3 subfamilies, but not from the RidA and Rid1 subfamilies deaminated iminoarginine, generated *in situ* by the *Pseudomonas aeruginosa* D-arginine dehydrogenase DauA. These data biochemically distinguished the subfamilies and showed Rid proteins have activity on a metabolite that is physiologically relevant in *Pseudomonas* and other bacteria.

3.2 INTRODUCTION

The Rid family is a group of small, sequence diverse proteins that has been divided into eight subfamilies by phylogenetic and bioinformatics analyses (1, 2). The archetypal RidA subfamily is highly conserved and present in all domains of life, where its importance is reflected by its broad conservation and the diverse consequences caused by its absence. RidA has been

implicated in various processes in a number of organisms including bacteria (3-7), plants (8, 9), fungi (10-12), and humans (13-15). The processes impacted by the relevant RidA member include nutrition (9, 11, 16), mitochondrial maintenance (10-12), development, and carcinogenesis (13, 17, 18). The *ridA* mutant phenotypes have been mechanistically defined in *Salmonella enterica* where they result from the inactivation of pyridoxal 5'-phosphate (PLP)-dependent enzymes by accumulation of 2-aminoacrylate (2AA) (6, 19-21).

The biochemical role of RidA has been characterized primarily in the context of specific PLP-dependent enzymes that proceed through 2-aminoacrylate (2AA), which spontaneously tautomerizes in solution to 2-iminopropionate prior to hydrolysis to a stable ketoacid. In the context of PLP-dependent enzymes, RidA increases the rate of 2AA conversion to pyruvate by facilitating hydrolysis, which is otherwise mediated by solvent water (Fig 3.1) (9, 22, 23). Members of the RidA subfamily from bacteria, plants, archaea, house dust mite, and humans had activity that was indistinguishable from the *S. enterica* protein when tested *in vivo* and *in vitro* (7, 9, 22, 24). Significantly, high resolution structures of more than twenty RidA homologs had been determined prior to any biochemical information on their function (1, 15, 25-30), many of which exist as Protein Data Bank entries yet to be published.

Unlike the RidA subfamily, which spans the three domains, subfamilies Rid1-Rid7 are found exclusively in prokaryotes (2). Bacterial genomes often encode multiple members of this protein superfamily suggesting broad, non-redundant involvement in metabolic processes of prokaryotes. For instance, the *S. enterica* genome encodes RidA, in addition to YoaB and STM1549, which are members of subfamily Rid2 and Rid7, respectively. The genome of *Escherichia coli* encodes five putative Rid proteins, two of the RidA subfamily, one of the Rid2 subfamily, a Rid7 subfamily protein and RutC (7). Other representative organisms with

numerous Rid family members include *Pseudomonas aeruginosa*, with nine Rid proteins, *Streptomyces coelicolor*, with eleven, and various *Bradyrhizobia* that encode up to sixteen putative Rid proteins (2).

Members of the Rid family are universally annotated as imine deaminases, yet beyond the RidA subfamily, there is a single report of activity for a Rid2 subfamily member (2), and to our knowledge no phenotype has been attributed to the lack of any member of a Rid1-7 subfamily. Based on bioinformatics, protein structures, and activity of RidA variant proteins, the residue analogous to RidA Arg-105 was suggested to be necessary and sufficient to predict deaminase activity (2, 22). Based on this criterion, Rid1, 2 and 3 subfamily members are expected to have deaminase activity, while subfamilies Rid4, 5, 6, and 7 are not. Thus far, this prediction is supported by a single isolate of Rid2, and a single isolate of Rid7 (YoaB and STM1549 of *S. enterica*, respectively) (2).

This study was initiated to validate the broad functional annotation of the Rid superfamily and extend our understanding of the Rid subfamilies beyond the RidA subfamily. Both *in vitro* assays and *in vivo* complementation of a *ridA* mutant were used to assess deaminase activity and substrate specificity of six proteins spanning subfamilies Rid1, Rid2 and Rid3. Iminoarginine deaminase was identified as an activity unique to proteins of the Rid2 and Rid3 subfamilies. In total, these studies bolstered the annotation of the Rid superfamily and suggested a role for Rid family members in facilitating metabolic steps that involve unstable intermediates.

3.3 MATERIALS AND METHODS

Bacterial strains, plasmids and media

Strains of *Salmonella enterica* were derivatives of serovar Typhimurium LT2 and are described, with relevant plasmids, in Table 1. *Escherichia coli* BL21AI was used for protein overexpression. Primers used to generate plasmids are listed in Table 2. pDM1474 (pBAD24-ACIAD3089) was synthesized by GenScript (Piscataway, NJ) and the coding sequence was codon optimized for expression in *Escherichia coli*. Other plasmids were constructed by amplifying the appropriate gene from genomic DNA, and ligating into a modified pBAD24 vector (pCV1) (31) or into pET28 using BspQI (32). Plasmid constructs were verified by sequence analysis and sequencing was performed by Genewiz (South Plainfield, NJ) or Eton Bioscience Inc. (San Diego, CA).

Difco Nutrient Broth (8 g/ liter) with NaCl (5 g/ liter) was used as rich medium for *S. enterica* and Lysogeny Broth (LB) was used for *E. coli*. Cells were grown in super broth (32 g/ liter tryptone, 20 g/ liter yeast extract, 5 g/ liter NaCl and 0.2 g/ liter NaOH) for protein overexpression. Difco BiTek agar was added (15 g/ liter) for solid medium. No-carbon E medium (NCE) supplemented with MgSO₄ (1 mM) (34), trace minerals (35) and glycerol (30 mM) was used as minimal medium. When indicated, compounds were added at the following concentrations: serine, 5 mM; cysteine, 250 μM; L-arabinose, 0.2%; ampicillin, 150 mg/ liter; kanamycin, 50 mg/ liter. Unless stated otherwise, chemicals were purchased from Sigma Aldrich (St. Louis, MO). Crystalline L-amino acid oxidase (A9253 Type I, dried venom *Crotalus adamanteus*), D-amino acid oxidase from porcine kidney (A5222) and catalase from bovine liver (C9322) were hydrated and stored at 4°C. Tris base was purchased from Amresco (Solon, OH).

Growth analysis

Liquid growth analyses were done in a 96-well microtiter plate using a BioTek Elx808 plate reader with continuous shaking (set to slow on the machine). *S. enterica* strains grown overnight in rich medium (2 mL) were used to inoculate the indicated minimal medium (1% inoculum). Growth was monitored as optical density (OD) at 650 nm for strains grown in biological triplicate at 37°C with shaking. Growth curves were plotted using GraphPad Prism (version 6.0g).

Protein purification

Rid proteins were purified from *E. coli* strain BL21AI harboring the appropriate pET construct. RidA protein was purified as described previously (36). Proteins PA0814, ACIAD3089, PA5083, PSPTO_0102, PFL_1385 and PSPTO_3006 were purified based on the RidA protocol with modifications of buffers and time of induction as needed to maximize protein recovery. All proteins were polyhistidine-tagged and were purified by nickel-affinity chromatography. Overnight cultures in LB (10 mL) were used to inoculate two flasks containing super broth (1.5 L) supplemented with kanamycin. Cultures were grown at 37°C with shaking until they reached an OD₆₅₀ greater than 0.7. Arabinose (0.1% w/v for PFL_1385 or 0.2% w/v for all other proteins) was added to induce T7 RNA polymerase production and cultures were shifted to 22°C (PA5083), 28°C (RidA), 30°C (PA0814, ACIAD3089, PSPTO_0102, or PFL_1385) or 37°C (PSPTO_3006) for 18 hours. Cells were harvested by centrifugation at 7,000 x g and the pellets were stored at -80°C until use. Binding buffer (50 mM potassium phosphate pH 7.5, 100 mM NaCl, 5 mM imidazole, and 10% glycerol) was added to thaw cells (2 mL per gram wet cell weight) along with lysozyme (1 mg/ mL) and DNase (20 Units/ mL), and the cells were lysed with a French pressure cell. The lysate was clarified by centrifugation (40,000 x g for

45 minutes) and passed through a 0.45 μ m syringe filter (Argos Technologies) prior to being loaded onto a 5 mL Ni-NTA FPLC column of HisPur resin (ThermoFisher Scientific) on an ÄKTA FPLC equipped with a UV lamp and Frac920 fraction collector. Protein was eluted with a 0-100% gradient of elution buffer (50 mM potassium phosphate pH 7.5, 100 mM NaCl, 500 mM imidazole and 10% glycerol) per the column manufacturer's instructions (GE Healthcare). Elution of polyhistidine-tagged proteins was monitored by absorbance at 280 nm over the gradient and occurred between 20-80% elution buffer. Fractions containing desired protein (based on UV absorption spectroscopy, SDS-PAGE gel analysis) were pooled and concentrated with a 7,000 molecular weight cut-off protein concentrator (Millipore). The preparations were dialyzed into storage buffer (50 mM potassium phosphate pH 7.5, 10% glycerol) using a PD-10 desalting column (GE Healthcare). Proteins (0.125-2 μ g) were subjected to SDS/PAGE and purity was assessed using a Foto/Analyst FX (Fotodyne) imager and TotalLab Quant v11 densitometry software. Purified PFL_1835 was enriched to greater than 90% purity, while all other Rid proteins used in this study were enriched to greater than 98% purity. Protein concentration was quantified using BCA Protein Assay (Thermo Scientific), and the samples were frozen in liquid nitrogen and stored at -80°C.

DauA was partially purified from DM15625, a BL21AI strain harboring pET28-*dauA*. An overnight culture (10 mL) grown in LB containing kanamycin was used to inoculate 1.5 liters of super broth containing kanamycin. A total culture volume of 3 liters was grown at 37°C until the cultures reached an OD₆₅₀ of 0.6. L-arabinose (3 grams) was added to each flask, and the temperature was shifted to 18°C for 16 additional hours. A sample (5 mL) was pelleted, resuspended in buffer (1 mL of 50 mM potassium phosphate pH7.5, 1 mM EDTA), sonicated, and activity confirmed using a dye-based crude extract dehydrogenase assay (37). Cells were

harvested by centrifugation at 7,000 x g and the pellet (24.5 grams) was stored at -80°C. The cell pellet was thawed with bind buffer (20 mM sodium phosphate pH 7.4, 300 mM sodium chloride, 10 mM imidazole) containing 1 mM PMSF, lysozyme (1 mg/ mL) and DNase (20 Units/ mL). Cells were lysed using a French pressure cell and the lysate was clarified using the methods described above. The cell-free extract was loaded onto a HisPur Ni-NTA resin (1 mL, Thermo Scientific, Rockford, IL) gravity column, washed twice with wash buffer (20 mM sodium phosphate pH 7.4, 300 mM sodium chloride, 25 mM imidazole) and eluted with elution buffer (20 mM sodium phosphate pH 7.4, 300 mM sodium chloride, 250 mM imidazole). The protein sample was dialyzed into storage buffer (50 mM potassium phosphate pH 7.5, 10% glycerol), quantified using BCA Protein Assay (Thermo Scientific), and frozen in liquid nitrogen and stored at -80°C. A sample was evaluated for purity using SDS-PAGE analysis as described for Rid protein, and was found to be 60% pure. The DauA-enriched sample generated imines that were derivatized by semicarbazide and detected within the linear range of the spectrophotometer (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA).

Enzyme assays

All assays were performed at room temperature (~25°C).

Cysteine desulphydrase CdsH

A coupled assay using cysteine desulphydrase-dependent pyruvate formation and the oxidation of NADH by lactate dehydrogenase was used to assess Rid activity as previously described (23). The assay contained Tris base (100 mM, pH 8), NADH (0.25 mM), pyridoxal 5'-phosphate (30 μ M) and pyruvate kinase/lactate dehydrogenase (5 Units). Purified Rid proteins and cysteine desulphydrase (CdsH) were added at monomeric concentrations of 0.19 μ M and 0.27 μ M, respectively. Reactions were started by the addition of a freshly prepared L-cysteine

stock to a range of final concentrations between 0.1 and 10 mM, and absorbance at 340 nm was measured over time. Rates represent initial velocities obtained in the first 30 seconds following substrate addition and moles of pyruvate formed were calculated using the molar extinction coefficient of NADH at 340 nm ($\epsilon = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$).

L-amino acid oxidase

The LOX-based assay for Rid activity was adapted from a previously described continuous assay (38). Simply, the imine intermediates were derivatized with semicarbazide resulting in semicarbazone compounds that were detected by absorbance at 248 nm (2). The assay mixture (100 μL total volume) contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), bovine liver catalase (24 Units), L-amino acid oxidase (0.4 μM) and 10 μM Rid protein. Reactions were started with the addition of an L-amino acid or 2-aminobutyrate to the final concentration indicated. Following the addition of substrate, the path length for each well was measured and used along with the molar extinction coefficient for semicarbazone ($\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$) to calculate the rate of semicarbazone formation.

3.4 RESULTS AND DISCUSSION

Proteins from Rid1, 2 and 3 subfamilies deaminate 2AA *in vitro*

Representative members of Rid1, Rid2, and Rid3 subfamilies, predicted to have deaminase activity due to the presence of the relevant arginine residue, were chosen for study. Rid1 representatives were from *Pseudomonas aeruginosa*, and *Acinetobacter baylyi*. Members of Rid2 came from *S. enterica*, *Pseudomonas aeruginosa*, and *Pseudomonas syringae*, and members from Rid3 came from *Pseudomonas syringae* and *Pseudomonas fluorescens*. Source organism, locus tag and sequence alignment of each protein are shown in Fig 2 and Table 3.

Growth defects of a *ridA* mutant have been attributed to the accumulation of 2-aminoacrylate *in vivo* (6, 19-21, 41). The standard *in vitro* assay for RidA activity uses a PLP-dependent dehydratase to generate 2-aminoacrylate *in situ*. *In situ* generation of the 2AA substrate is necessary due to its short (~1.5 s) half-life in aqueous solution (42). In this assay, Rid activity is detected as an increased rate of pyruvate formation (from substrate serine) compared to that of a reaction mixture devoid of Rid protein, where deamination is catalyzed solely by solvent water. RidA can deaminate a wide range of substrates, but only 2AA (derived from serine or cysteine) and 2 aminocrotonate (derived from threonine) have been shown to be physiologically relevant (2, 22). Six Rid proteins (Table 3) were tested for the ability to deaminate 2AA *in vitro* using cysteine desulfhydrase (EC 2.5.1.47, CdsH) as the *in situ* generator. The data in Fig 3 showed all proteins accelerated the rate of pyruvate formation in a coupled assay following NADH oxidation by lactate dehydrogenase. These data indicated that the proteins could deaminate 2AA and strengthened the conclusion that Rid superfamily members with the relevant arginine residue (RidA Arg-105) had deaminase activity.

Rid proteins functionally complement a *ridA* mutant

S. enterica strains lacking *ridA* have a growth defect in minimal medium containing 5 mM serine or 250 μ M cysteine due to the accumulation of 2AA (16, 23, 43). These growth defects were used to assess *in vivo* function of representative members of the subfamilies Rid1-3. Rid-protein encoding genes were cloned into pBAD24 vectors, putting their expression under the control of arabinose. Each of the plasmids, in addition to control plasmids pDM1439 and pBAD24, was transformed into strain DM12920 (*ridA*). The resulting strains were grown in minimal medium containing glycerol (30 mM) as the sole carbon source with the addition of serine (Fig 4 panels A-D) or cysteine (Fig 4 panels E-H). Several points were noted in the growth

data. First, the control plasmid pDM1439 restored growth in serine and cysteine with and without induction of *ridA* by arabinose (panels A, E). This result indicated that low expression of the *ridA* gene from the pBAD plasmid without induction (presumed to be lower than with the other genes with induction), was sufficient to complement the loss of the chromosomal locus. Second, four of the six remaining plasmids supported growth of the *ridA* mutant when the respective genes were induced by arabinose (panels B-D, F-H).

Finally, protein ACIAD3089 from *A. baylyi* supported growth of the *ridA* mutant, without induction, when cysteine was present (panel F), but required induction to allow growth in the presence of serine (panel B). Significantly, the pattern of complementation did not correlate with subfamily assignment of the proteins. It was formally possible that some (or all) of the negative results *in vivo* were due to instability of the gene products, which was not specifically tested here. However, the ability to purify the relevant proteins in active forms appeared to minimize this possibility.

Proteins from Rid1, 2, 3 subfamilies have different substrate specificities

Imine deaminase activity of the six Rid proteins was quantified using L-amino acid oxidase (LOX) in an assay described in a previous study, which used proteins RidA, YoaB (Rid2), and STM1549 (Rid7) from *S. enterica* (2). LOX is a promiscuous enzyme that generates imines *in situ* from a range of L-amino acids using an FAD-dependent mechanism. In an assay mixture with Rid proteins included, the resulting imines have three fates that are depicted in Figure 5, i) they can react with semicarbazide to form a semicarbazone that absorbs at 248 nm, ii) they can be converted by solvent water into the corresponding α -ketoacid, or iii) they can be converted by a Rid protein to the α -ketoacid. In this assay, the rate of semicarbazone formation is inversely proportional to Rid activity since the reduction in semicarbazone formation

corresponds to an increase in Rid-dependent α -ketoacid formation (9). Each of the six putative Rid proteins tested slowed the rates of semicarbazone formation in reaction mixtures with a variety of amino acid substrates of LOX as the source of the reactive imine intermediate (Table 4, reaction mixtures 2-8 vs 1). Reaction mixture #1 did not contain a Rid protein and was used to quantify imine derivatization when α -ketoacid formation was mediated only by solvent water. Reaction mixture #2 contained RidA and was used as positive control for activity, defined here as the ability to decrease imines available for derivatization by semicarbazide.

In total, the data in Table 4 supported the conclusion that the six proteins annotated as Rid family members had imine deaminase activity on multiple substrates. Beyond this general conclusion, a few observations stood out. First, RidA did not have significant activity with the imines derived from L-Phe and L-His, while the protein was active on the imines of L-Leu, L-Gln and L-Met. Second, the *A. baylyi* ACIAD3089 protein had a level of activity similar to RidA across substrates. With a few outliers, the remaining proteins, all from *Pseudomonads*, displayed activity on all substrates similar to or higher than, the RidA protein. A notable observation was that the non-RidA proteins had significantly more activity than RidA with the imine derived from L-Phe and L-His. The significance of this substrate specificity is unclear, but it may hint at a physiological role of Rid proteins.

Deamination of iminoarginine separates Rid subfamilies

When arginine was used as a LOX substrate, representatives from the Rid1, Rid2 and Rid3 subfamilies appeared to have more deaminase activity than RidA (data not shown). This activity distribution piqued our interest due to the presence of iminoarginine in the metabolism of *Pseudomonas*, the source of the relevant enzymes.

In *P. aeruginosa*, L-arginine is generated in a racemization pathway that includes D-Arg dehydrogenase (a.k.a. D-Arg:acceptor oxidoreductase (deaminating), EC 1.4.99.6, DauA), to convert D-Arg to 5-guanidino-2-ketopentanoate via an iminoarginine (5-carbamimidamido-2-iminopentanoate) intermediate (44-46). Given that iminoarginine is an intermediate of the DauA-catalyzed reaction, it was possible that Rid proteins could accelerate the deamination of iminoarginine *in vivo*. Such a scenario would require the release of the iminoarginine from the DauA active site to make it available for the Rid proteins. This possibility was addressed *in vitro* with purified DauA, monitoring semicarbazone formation with a strategy similar to that of the LOX assay. In the absence of a Rid protein, control reactions were initiated with D-Arg (1 mM) and showed the rate of semicarbazone product formation was linear over a DauA protein concentration between 0.3-6 μ M (data not shown). The effect of Rid proteins on the rate of semicarbazone formation was determined and the data are reported as a change in absorbance at 248 nm as a function of time (Fig 6A), or as the rate of semicarbazone formation (Fig 6B). As shown in Fig 6, members of the Rid2 subfamily (PSPTO_0102, PA5083) and the Rid3 subfamily (PFL_1385, PSPTO_3006) significantly decreased the rate of semicarbazone formation indicating these proteins used iminoarginine as a substrate. In contrast, neither RidA, nor the Rid1 subfamily proteins PA0814 or ACIAD3089 had significant activity in this assay. These results identified an activity unique to Rid2 and 3 subfamilies. In addition, the results allowed the conclusion that the DauA enzyme released iminoarginine from the active site, where it was then available as substrate for Rid proteins. It is formally possible that the Rid2 and Rid3 proteins are interacting with DauA. Based on RidA precedent and the ability of these Rid proteins to act on iminoarginine produced by eukaryotic enzymes in addition to bacterial DauA, we consider this

possibility unlikely. These data defined the third bacterial enzyme mechanism to be associated with Rid function.

In additional assays, arginine concentration was held constant at 1 mM and the concentration of the relevant four Rid proteins was titrated. The data showed that as Rid protein concentration decreased, the rate of semicarbazone formation increased (Fig 6C). These data demonstrated that the fate of the imine depended upon Rid protein concentration, as expected for a process in which the protein was competing with the semicarbazide for the available iminoarginine.

3.5 CONCLUSIONS

Results presented here have expanded our understanding of the activities catalyzed by Rid family members beyond RidA. Each of the six proteins studied, representing three Rid subfamilies, had deaminase activity on a range of substrates *in vitro*. These data validated the annotation of the Rid superfamily, and supported the scenario in which the active site arginine residue analogous to RidA Arg-105 was the determinant of deaminase activity.

Activity in *in vitro* assays has the caveat that it may or may not represent a physiologically relevant role of the protein. For this reason, the finding that Rid2 and Rid3 subfamilies were active on the iminoarginine generated by the DauA enzyme was significant. First, DauA has a central metabolic role in *Pseudomonas*, the organismal source of several of the Rid proteins tested. Second, the ability of Rid proteins to compete with semicarbazide for the iminoarginine product showed that the imine was released from the DauA enzyme active site. With these data, DauA became the third defined bacterial reaction mechanism to use either a Rid protein or solvent water to generate a ketoacid, the required substrate for a subsequent enzymatic step. This result led to the hypothesis that a Rid protein(s) participates in the pathway of D-

arginine racemization *in vivo* (Fig 7). While in a simple scenario, blocking the relevant Rid protein would result in the inability to racemize arginine, this is not likely to be the case. The redundancy of Rid activity with that of solvent water means that Rid proteins facilitate a reaction that occurs, albeit slowly, in the cell. This makes demonstrating an *in vivo* role challenging since loss of the Rid protein is expected to generate a subtle pathway defect, and may not produce a clear phenotype. In fact, the role of RidA was uncovered not because a product was produced more slowly, but because the accumulated substrate (i.e., 2-aminoacrylate) had detrimental effects. We hypothesize that the general role of Rid proteins is to facilitate reactions that are limited by the rate of hydrolysis allowed by water, and thus make a variety of metabolic pathways more efficient. In total, efficiency provided by Rid proteins, and other potential metabolic modulators, would increase overall organismal fitness enough to explain the selective pressure that maintained this superfamily as reflected in its broad conservation.

Despite their role in overall fitness, identifying specific pathways that produce substrates for Rid proteins is hampered by the expectation that any growth defects would be subtle. Defining the physiological roles of Rid proteins will require multiple approaches including creative phenotypic analysis, targeted biochemical approaches and a fair amount of serendipity. The results here provide a framework to explore the role of Rid proteins, specifically in the racemization of arginine in *P. aeruginosa*.

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3.7 REFERENCES

1. Burman JD, Stevenson CE, Sawers RG, Lawson DM. The crystal structure of *Escherichia coli* TdcF, a member of the highly conserved YjgF/YER057c/UK114 family. *BMC Struct Biol.* 2007;7:30.
2. Niehaus TD, Gerdes S, Hodge-Hanson K, Zhukov A, Cooper AJ, ElBadawi-Sidhu M, et al. Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC Genomics.* 2015;16:382.
3. Enos-Berlage JL, Langendorf MJ, Downs DM. Complex metabolic phenotypes caused by a mutation in yjgF, encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of bacteriology.* 1998;180(24):6519-28.
4. Browne BA, Ramos AI, Downs DM. PurF-independent phosphoribosyl amine formation in yjgF mutants of *Salmonella enterica* utilizes the tryptophan biosynthetic enzyme complex anthranilate synthase-phosphoribosyltransferase. *J Bacteriol.* 2006;188(19):6786-92.
5. Christopherson MR, Schmitz GE, Downs DM. YjgF is required for isoleucine biosynthesis when *Salmonella enterica* is grown on pyruvate medium. *J Bacteriol.* 2008;190(8):3057-62.
6. Flynn JM, Christopherson MR, Downs DM. Decreased coenzyme A levels in ridA mutant strains of *Salmonella enterica* result from inactivated serine hydroxymethyltransferase. *Mol Microbiol.* 2013;89(4):751-9.
7. Borchert AJ, Downs DM. The Response to 2-Aminoacrylate Differs in *Escherichia coli* and *Salmonella enterica*, despite Shared Metabolic Components. *J Bacteriol.* 2017;199(14).
8. Leitner-Dagan Y, Ovadis M, Zuker A, Shklarman E, Ohad I, Tzfira T, et al. CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. *Planta.* 2006;225(1):89-102.
9. Niehaus TD, Nguyen TND, Gidda SK, ElBadawi-Sidhu M, Lambrecht JA, McCarty DR, et al. Arabidopsis and Maize RidA Proteins Preempt Reactive Enamine/Imine Damage to Branched-Chain Amino Acid Biosynthesis in Plastids. *The Plant Cell.* 2014;26(7):3010-22.
10. Oxelmark EM, Antonio; Malanchi, Ilaria; Magherini, Francesca; Jaquet, Laurence; Hajibagheri, M. A. Nasser; Blight, Kenneth J.; Jauniaux, Jean-Claude; Tommassino, Massimo. Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome. *Molecular and cellular biology.* 2000;20(20):7784-97.

11. Kim J-MY, Hiroshi; Shirahige, Katsuhiko. A member of the YER057c/yjgF/Uk114 family links isoleucine biosynthesis to intact mitochondria maintenance in *Saccharomyces cerevisiae*. *Genes to cells*. 2001;6:507-17.
12. Accardi R, Oxelmark E, Jauniaux N, de Pinto V, Marchini A, Tommasino M. High levels of the mitochondrial large ribosomal subunit protein 40 prevent loss of mitochondrial DNA in null mmf1 *Saccharomyces cerevisiae* cells. *Yeast*. 2004;21(7):539-48.
13. Ceciliani F, Ludovica F, Negri A, Colombo I, Berra B, Bartorelli A, Ronchi S. The primary structure of UK114 tumor antigen. *FEBS Letters*. 1996;393:147-50.
14. Bussolati G, Geuna M Fau - Bussolati B, Bussolati B Fau - Millesimo M, Millesimo M Fau - Botta M, Botta M Fau - Bartorelli A, Bartorelli A. Cytolytic and tumor inhibitory antibodies against UK114 protein in the sera of cancer patients. *International Journal of Oncology*. 1997;10(1019-6439 (Print)):779-85.
15. Deriu D, Briand C, Mistiniene E, Naktinis V, Grutter MG. Structure and oligomeric state of the mammalian tumour-associated antigen UK114. *Acta crystallographica Section D, Biological crystallography*. 2003;59(Pt 9):1676-8.
16. Enos-Berlage JLL, Mark J.; Downs, Diana M. Complex metabolic phenotypes caused by a mutation in *yjgF* encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of Bacteriology*. 1998;180(24):6519-28.
17. Racca SDC, Francesco; Bartorelli, Alberto. Growth inhibition of DMBA-induced rat mammary carcinomas by UK 114. *Virchows Arch*. 1997(431):323-8.
18. Chong CL, Huang SF, Hu CP, Chen YL, Chou HY, Chau GY, et al. Decreased expression of UK114 is related to the differentiation status of human hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2008;17(3):535-42.
19. Flynn JM, Downs DM. In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5'-phosphate cofactor. *J Bacteriol*. 2013;195(16):3603-9.
20. Lambrecht JA, Schmitz GE, Downs DM. RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life. *MBio*. 2013;4(1):e00033-13.
21. Ernst DC, Downs DM, Metcalf WW. 2-Aminoacrylate Stress Induces a Context-Dependent Glycine Requirement in *iridA* strains of *Salmonella enterica*. *Journal of Bacteriology*. 2016;198(3):536-43.
22. Lambrecht JA, Flynn JM, Downs DM. Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions. *J Biol Chem*. 2012;287(5):3454-61.

23. Ernst DC, Lambrecht JA, Schomer RA, Downs DM. Endogenous Synthesis of 2-Aminoacrylate Contributes to Cysteine Sensitivity in *Salmonella enterica*. *Journal of Bacteriology*. 2014;196(18):3335-42.
24. ElRamlawy KG, Fujimura T, Baba K, Kim JW, Kawamoto C, Isobe T, et al. Der f 34, a Novel Major House Dust Mite Allergen Belonging to a Highly Conserved Rid/YjgF/YER057c/UK114 Family of Imine Deaminases. *J Biol Chem*. 2016;291(41):21607-15.
25. Volz K. A test case for structure-based functional assignment: The 1.2Å crystal structure of the YjgF gene product from *Escherichia coli*. *Protein Science*. 1999;8:2428-37.
26. Sinha S, Rappu P, Lange SC, Mantsala P, Zalkin H, Smith JL. Crystal structure of *Bacillus subtilis* YabJ, a purine regulatory protein and member of the highly conserved YjgF family. *Proc Natl Acad Sci U S A*. 1999;96(23):13074-9.
27. Deaconescu AM, Roll-Mecak A, Bonanno JB, Gerchman SE, Kycia H, Studier FW, et al. X-ray structure of *Saccharomyces cerevisiae* homologous mitochondrial matrix factor 1 (Hmf1). *Proteins*. 2002;48(2):431-6.
28. Parsons L, Bonander N, Eisenstein E, Gilson M, Kairys V, Orban J. Solution structure and functional ligand screening of HI0719, a highly conserved protein from bacteria to humans in the YjgF/YER057c/UK114 family. *Biochemistry*. 2003;42(1):80-9.
29. Manjasetty BA, Delbruck H, Pham DT, Mueller U, Fieber-Erdmann M, Scheich C, et al. Crystal structure of *Homo sapiens* protein hp14.5. *Proteins*. 2004;54(4):797-800.
30. Miyakawa T, Hatano KI, Lee WC, Kato Y, Sawano Y, Yumoto F, et al. Crystallization and preliminary X-ray analysis of the YjgF/YER057c/UK114-family protein ST0811 from *Sulfolobus tokodaii* strain 7. *Acta crystallographica Section F, Structural biology and crystallization communications*. 2005;61(Pt 9):828-30.
31. VanDrissse CM, Escalante-Semerena JC. New high-cloning-efficiency vectors for complementation studies and recombinant protein overproduction in *Escherichia coli* and *Salmonella enterica*. *Plasmid*. 2016;86:1-6.
32. Galloway NR, Toutkoushian H, Nune M, Bose N, Momany C. Rapid Cloning For Protein Crystallography Using Type IIS Restriction Enzymes. *Crystal Growth & Design*. 2013;13(7):2833-9.
33. Way JC, Davis MA, Morisato D, Roberts DE, Kleckner N. New Tn10 derivatives for transposon mutagenesis and for construction of lacZ operon fusions by transposition. *Gene*. 1984;32(3):369-79.
34. Vogel HJB, David M. Acetylornithinase of *Escherichia coli*: Partial purification and some properties. *Journal of Biological Chemistry*. 1955;218:97-106.

35. Balch W, E.; Wolfe, R. S. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Applied and Environmental Microbiology*. 1976;32(6):781-91.
36. Lambrecht JA, Browne BA, Downs DM. Members of the YjgF/YER057c/UK114 family of proteins inhibit phosphoribosylamine synthesis in vitro. *J Biol Chem*. 2010;285(45):34401-7.
37. Jann AM, Hideki; Haas, Dieter. The fourth arginine catabolic pathway of *Pseudomonas aeruginosa*. *Journal of general microbiology*. 1988;134:1043-53.
38. Hafner EWW, Daniel. Reactivity of the imino acids formed in the amino acid oxidase reaction. *Biochemistry*. 1979;18(3):411-7.
39. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*. 2011;7:539.
40. Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M, et al. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res*. 2005;33(17):5691-702.
41. Ernst DC, Anderson ME, Downs DM. L-2,3-diaminopropionate generates diverse metabolic stresses in *Salmonella enterica*. *Mol Microbiol*. 2016;101(2):210-23.
42. Hillebrand GGD, James L.; Suelter, Clarence H. Formation of an intermediate and its rate of conversion to pyruvate during the tryptophanase-catalyzed degradation of S-o-Nitrophenyl-L-cysteine. *Biochemistry*. 1979;18(9):1751-5.
43. Downs DM, Ernst DC. From microbiology to cancer biology: the Rid protein family prevents cellular damage caused by endogenously generated reactive nitrogen species. *Molecular Microbiology*. 2015;96(2):211-9.
44. Li C, Lu CD. Arginine racemization by coupled catabolic and anabolic dehydrogenases. *Proc Natl Acad Sci U S A*. 2009;106(3):906-11.
45. Yuan H, Fu G, Brooks PT, Weber I, Gadda G. Steady-state kinetic mechanism and reductive half-reaction of D-arginine dehydrogenase from *Pseudomonas aeruginosa*. *Biochemistry*. 2010;49(44):9542-50.
46. Yuan H, Xin Y, Hamelberg D, Gadda G. Insights on the mechanism of amine oxidation catalyzed by D-arginine dehydrogenase through pH and kinetic isotope effects. *J Am Chem Soc*. 2011;133(46):18957-65.

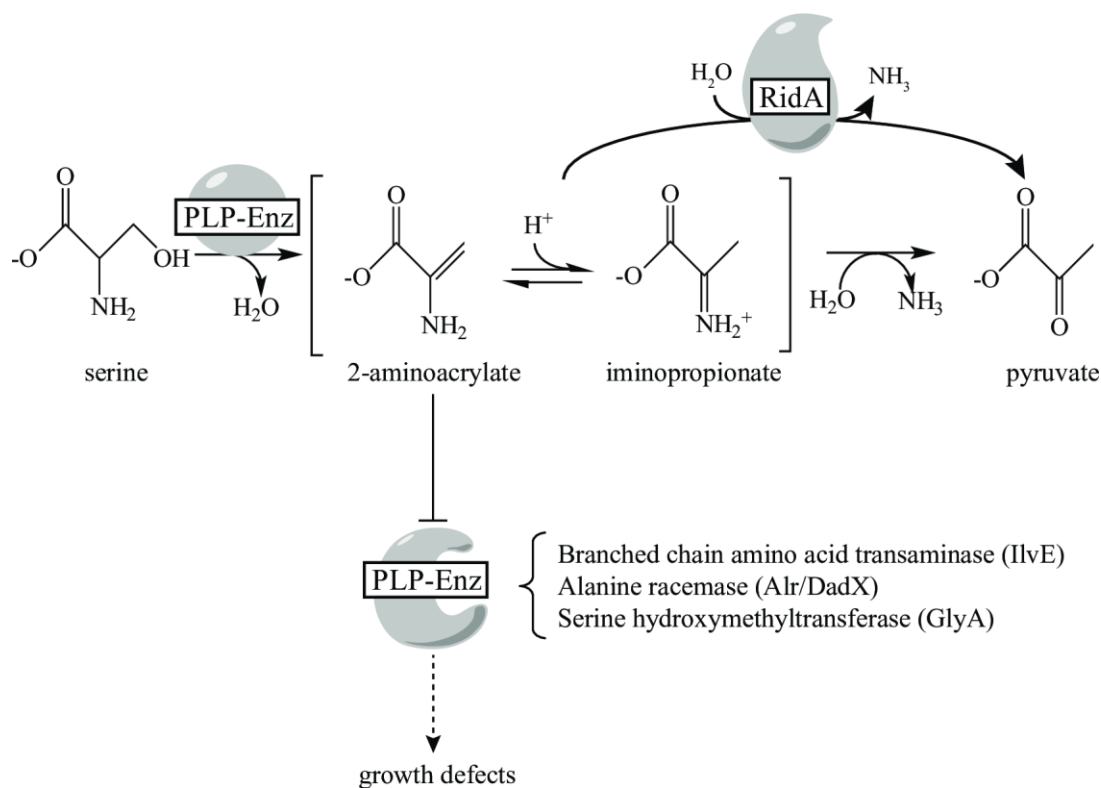


Figure 3.1 RidA activity *in vivo*

The PLP-dependent generation of the enamine 2 aminoacrylate from serine. Enamine/imine intermediates (2-aminoacrylate/imino propionate) are in equilibrium and the latter is hydrolyzed by solvent water or facilitated by RidA protein, resulting in production of pyruvate. 2AA accumulation in an *S. enterica ridA* mutant is responsible for the inactivation of particular PLP-enzymes, which leads to growth defects.

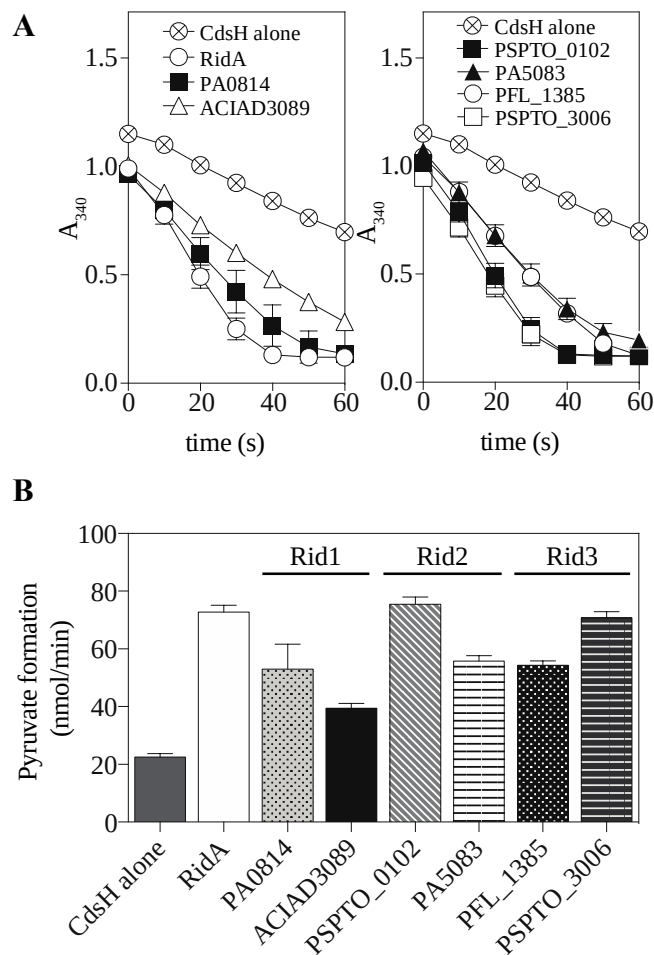


Figure 3.3 Rid proteins deaminate 2AA *in vitro*

Reaction mixtures (100 μ L) contained Tris-HCl (100 mM, pH 8), NADH (250 μ M), pyridoxal 5'-phosphate (30 μ M), pyruvate kinase/lactate dehydrogenase (5 Units) and purified cysteine desulphydrase CdsH (0.27 μ M). Purified Rid protein (0.19 μ M) was added as indicated. **(A)** Absorbance was monitored at 340 nm for 60 seconds following the addition of L-cysteine to 1 mM final concentration. Reactions contained CdsH and purified Rid proteins as indicated. **(B)** The initial rate of pyruvate formation for each reaction was calculated using the molar extinction coefficient ($\epsilon = 6,200 \text{ M}^{-1} \text{ cm}^{-1}$) for NADH oxidation during the first 30 seconds. The mean of three replicates is plotted and error bars indicate standard deviation.

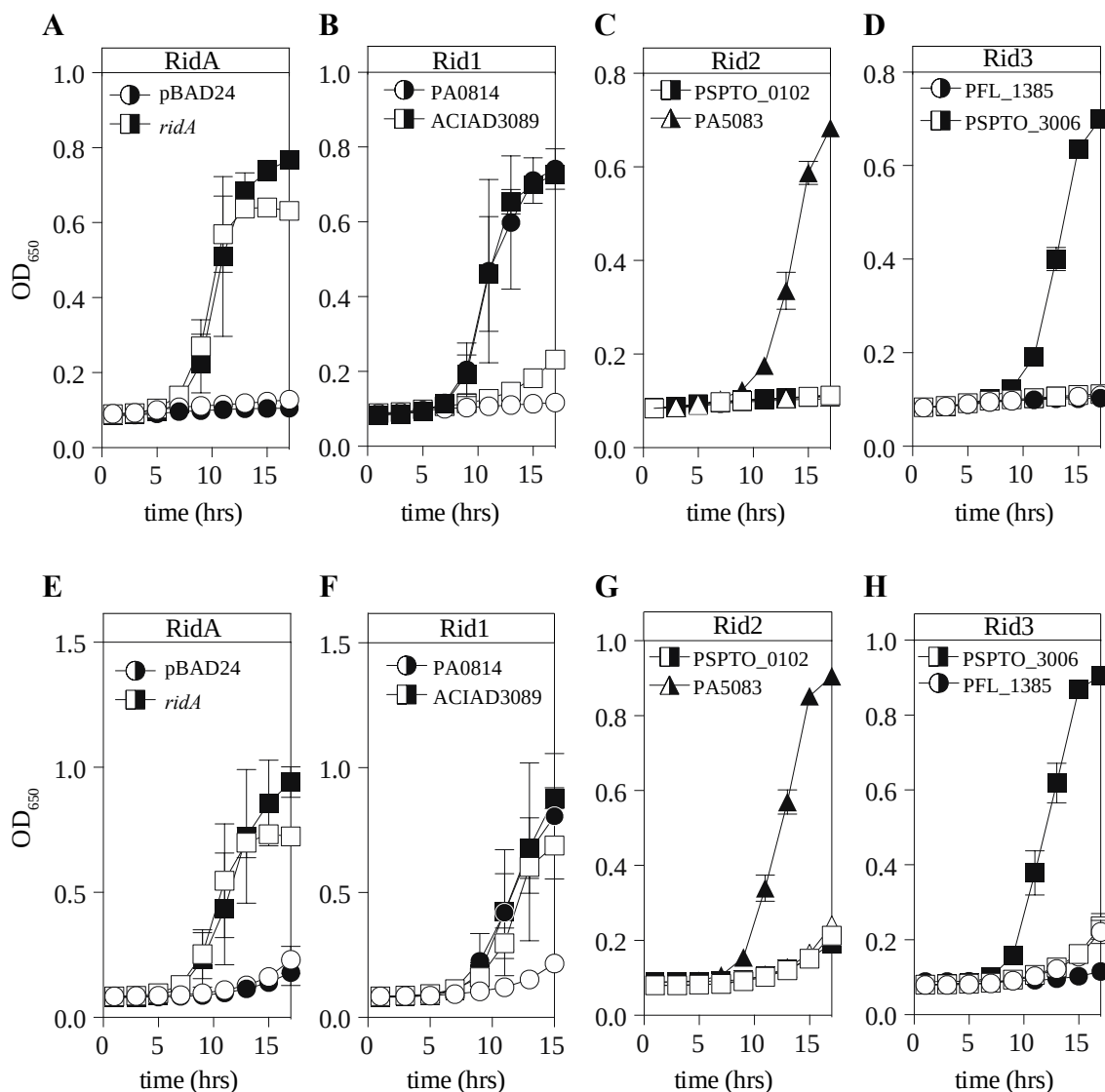


Figure 3.4 Representative RidA and Rid1 proteins rescue growth of *S. enterica* *ridA* mutant strain

An *S. enterica* *ridA* strain (DM12920) was transformed with pBAD24 constructs harboring no insert (pBAD24), *S. enterica* *ridA*, *P. aeruginosa* PA0814, *A. baylyi* ACIAD3089, *P. syringae* PSPTO_0102, *P. aeruginosa* PA5083, *P. fluorescens* PFL_1385, or *P. syringae* PSPTO_3006. The strains were grown in minimal glycerol medium supplemented with (A-D) 5 mM serine or (E-H) 250 μ M cysteine, with (closed symbols) or without (open symbols) arabinose. The corresponding Rid subfamily assignment is presented above each graph. Growth was monitored by optical density at 650 nm with shaking at 37°C. Error bars indicate standard deviation for three biological replicates.

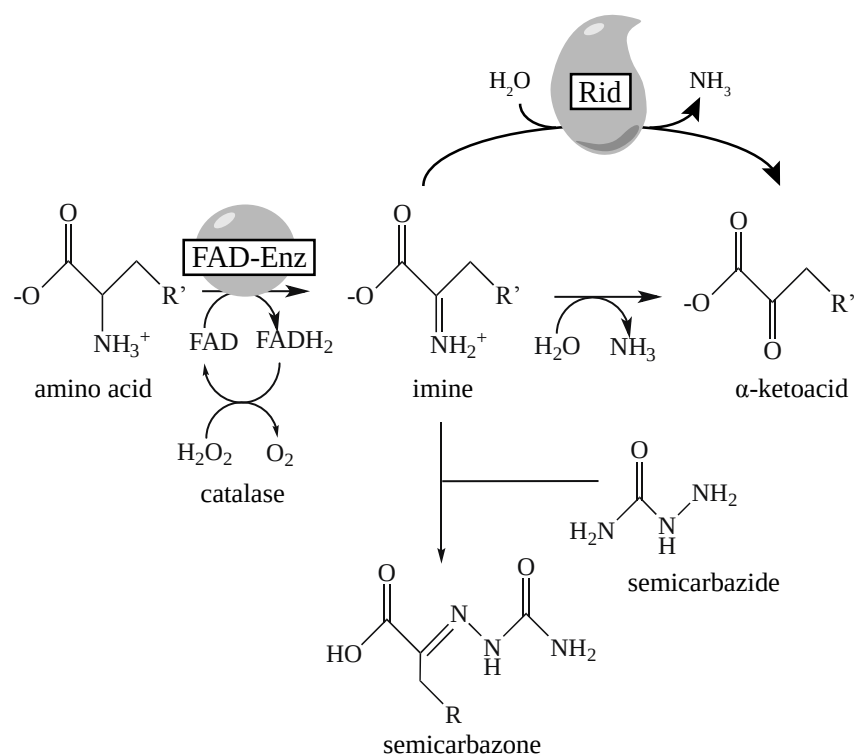


Figure 3.5 Reaction schemes for FAD-dependent oxidase

The reaction mechanism shows the FAD-dependent production of an imine from an amino acid. Imines are hydrolyzed by solvent water or facilitated by Rid proteins, or in the presence of semicarbazide react to form a semicarbazone compound. R' represents the amino acid functional group.

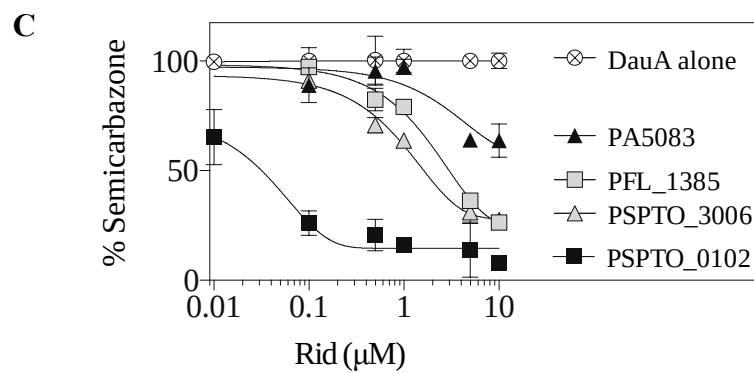
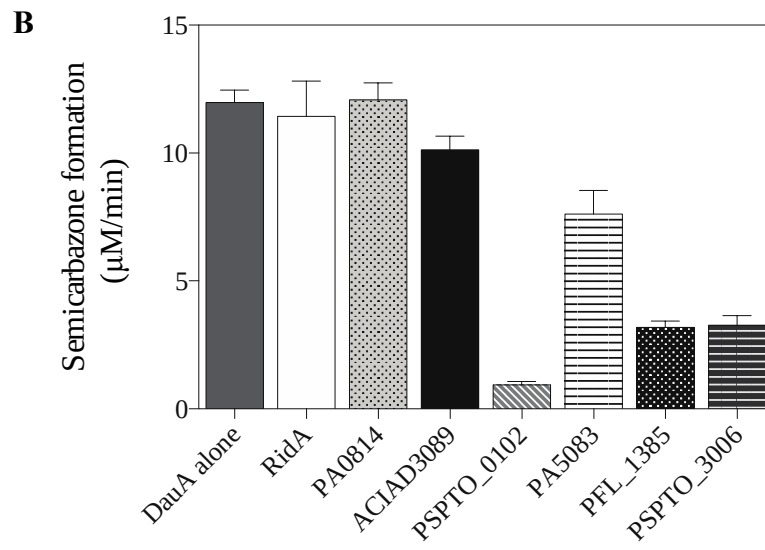
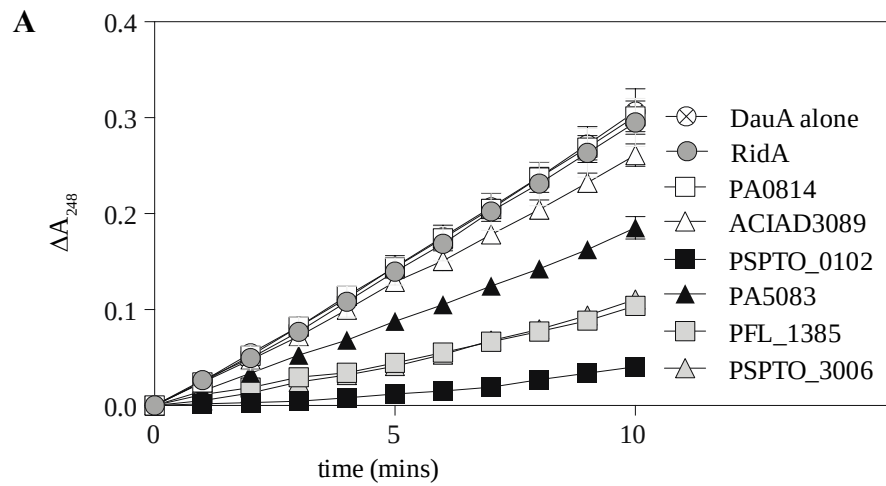


Figure 3.6 Rid2 and Rid3 proteins deaminate iminoarginine produced by DauA

Assay mixtures (100 μ L) were monitored in microtiter plate format and contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), bovine liver catalase (24 Units) and $\sim$ 1 μ M DauA (1.7 μ M total protein) from the partial purification. **(A)** Rid protein (10 μ M) was added and the substrate was D-arginine (1 mM). Following the addition of substrate, the path length of each well was measured and the change in absorbance at 248 nm was monitored for ten minutes. **(B)** The bar graph shows the rate of semicarbazone formation calculated from the observed rate of product formation at 248 nm using the molar extinction coefficient ($\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$). **(C)** The rate of semicarbazone formation is presented as a percentage of the rate observed in the control (DauA alone) reaction mixture that lacks Rid proteins. All reaction mixtures contained $\sim$ 1 μ M DauA and were initiated with 1 mM D-arginine. The effect of purified Rid2 and Rid3 protein concentration on the rate is shown. Error bars indicate standard deviation of three replicates and curves were fitted using the equation for one phase exponential decay using Prism.

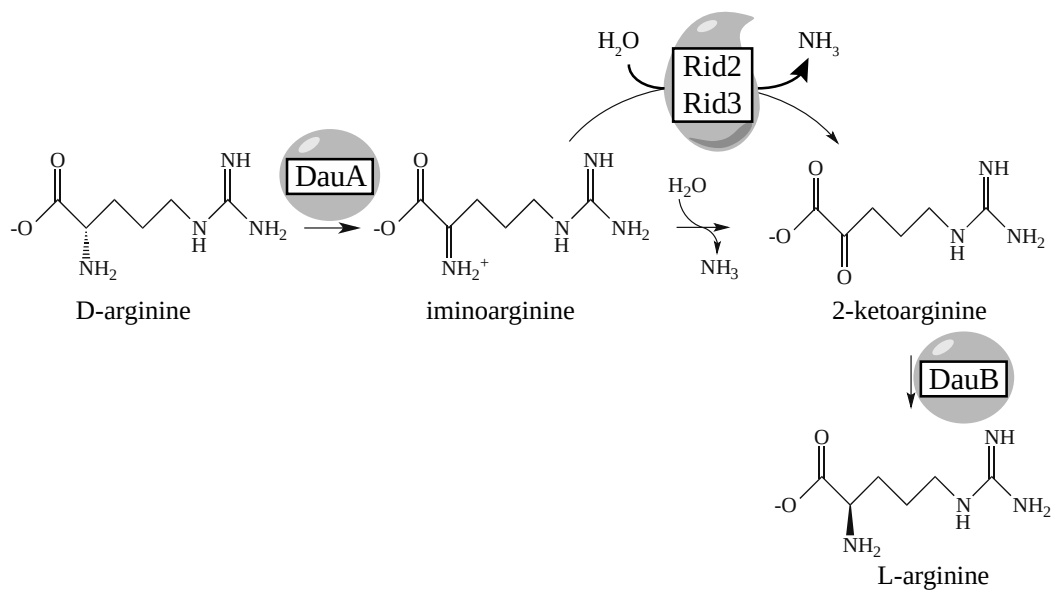


Figure 3.7 Working model for Rid2 and Rid3 activity

The FAD-dependent D-arginine dehydrogenase generates iminoarginine, which is deaminated by solvent water or by Rid proteins (Rid2 or Rid3), resulting in 2-ketoarginine formation. In *Pseudomonas aeruginosa*, DauB converts 2-ketoarginine to L-arginine, enabling the organism to grow on D-arginine as the sole carbon and nitrogen source (44).

Table 3.1 Strains and plasmids

Strain	Relevant genotype ^a	Encodes	Derivative of
<i>S. enterica</i> strains ^b			
DM12920	<i>ridA1::Tn10d(Tc)</i>		pBAD24
DM14846	<i>ridA1::Tn10d(Tc)</i> pDM1439	<i>S. enterica</i> RidA	pBAD24
DM14847	<i>ridA1::Tn10d(Tc)</i> pBAD24		pBAD24
DM15406	<i>ridA1::Tn10d(Tc)</i> pDM1464	<i>P. aeruginosa</i> PA0814	pBAD24
DM15407	<i>ridA1::Tn10d(Tc)</i> pDM1465	<i>P. fluorescens</i> PFL_1385	pBAD24
DM15482	<i>ridA1::Tn10d(Tc)</i> pDM1474	<i>A. baylyi</i> ACIAD3089	pBAD24
DM15486	<i>ridA1::Tn10d(Tc)</i> pDM1476	<i>P. syringae</i> PSPTO_0102	pBAD24
DM15440	<i>ridA1::Tn10d(Tc)</i> pDM1473	<i>P. syringae</i> PSPTO_3006	pBAD24
DM15687	<i>ridA1::Tn10d(Tc)</i> pDM1515	<i>P. aeruginosa</i> PA5083	pBAD24
<i>E. coli</i> strains ^c			
DM12740	pET20b- <i>ridA</i>	<i>S. enterica</i> RidA	pET20b
DM15324	pDM1459	<i>P. aeruginosa</i> PA0814	pET28
DM15325	pDM1460	<i>P. fluorescens</i> PFL_1385	pET28
DM15488	pDM1478	<i>P. syringae</i> PSPTO_3006	pET28
DM15489	pDM1477	<i>P. syringae</i> PSPTO_0102	pET28
DM15564	pDM1488	<i>A. baylyi</i> ACIAD3089	pET28
DM15625	pDM1496	<i>P. aeruginosa</i> DauA	pET28
DM15686	pDM1514	<i>P. aeruginosa</i> PA5083	pET28
Plasmid	Description or relative genotype		
pBAD24	P_{araBAD} expression vector, <i>bla</i> ⁺ (31) (pCV1)		
pET20b	T7 promoter, C-terminal His ₆ overexpression vector, <i>bla</i> ⁺ (Novagen)		
pET28	T7 promoter, C-terminal His ₆ overexpression vector, <i>kan</i> ⁺ (32)		

^a Tn10d(Tc) refers to the transposition-defective mini-Tn10(Tn10Δ16Δ17) (33)

^b *Salmonella enterica* strains are derivatives of *Salmonella typhimurium* serovar LT2

^c *Escherichia coli* strains are derivatives of BL21AI

Table 3.2 Primers

Primer name/ relevant plasmid	Primer sequence
PR501 (pDM1474 F)	NNGCTCTTCNTTCATGGTGAATAGAGATGATGCCTTTCTAA
PR502 (pDM1474 R)	NNGCTCTTCNTTATCACTCCTGCCATTATTTTG
PR503 (pDM1464 F)	NNGCTCTTCNTTCATGCAGACATCCCCCGCTCCG
PR504 (pDM1464 R)	NNGCTCTTCNTTATCATGGCCGCACCGCCGC
PR505 (pDM1465 F)	NNGCTCTTCNTTCATGTCTTTGAAAAGCACCGTCGTAG
PR506 (pDM1465 R)	NNGCTCTTCNTTATCAGCGCGCTCGGGC
PR507 (pDM1473 F)	NNGCTCTTCNTTCATGAGCGACAGCATTCAACGC
PR508 (pDM1473 R)	NNGCTCTTCNTTATCACGCGGATCTGGCCGCCA
PR511 (pDM1459 F)	NNGCTCTTCNATGCAGACATCCCCCGCTCCG
PR512 (pDM1459 R)	NNGCTCTTCNGTGTGGCCGCACCGCCGC
PR513 (pDM1460 F)	NNGCTCTTCNATGTCTTTGAAAAGCACCGTCGTAG
PR514 (pDM1460 R)	NNGCTCTTCNGTGGCGCGCTCGGGC
PR515 (pDM1478 F)	NNGCTCTTCNATGAGCGACAGCATTCAACGC
PR516 (pDM1478 R)	NNGCTCTTCNGTGCGCGGATCTGGCCGCCA
PR650 (pDM1476 F)	NNGCTCTTCNTTCATGTCAATCCAGCGCCAG
PR651 (pDM1476 R)	NNGCTCTTCNTTATTACGGCAGCGCGGCAATAA
PR652 (pDM1477 F)	NNGCTCTTCNATGTCAATCCAGCGCCAG
PR653 (pDM1477 R)	NNGCTCTTCNGTGCGGCAGCGCGGCAATAA
PR725 (pDM1496 R)	NNGCTCTTCNTTATCAGGGGGACAGGCGGCG
PR726 (pDM1496 F)	NNGCTCTTCNTTCATGATCGAAGCGGATTACCTCGT
PR729 (pDM1515 F)	NNGCTCTTCNTTCATGGAACCGACCCGTATCG
PR730 (pDM1515 R)	NNGCTCTTCNTTATCAGCCCCTGGCCGCCAC
PR731 (pDM1514 F)	NNGCTCTTCNATGGAACCGACCCGTATCGCC
PR732 (pDM1514 R)	NNGCTCTTCNGTGGCCCCTGGCCGCCACCACG

Table 3.3 Rid subfamily representatives and identity to *S. enterica* RidA

Subfamily ^a	Locus tag	Organism	Identity (%) ^b	Rid Proteins/genome ^c
Rid1	PA0814	<i>Pseudomonas aeruginosa</i> PAO1	26	9
	ACIAD_3089	<i>Acinetobacter baylyi</i> ADP1	23	7
Rid2	PSPTO_0102	<i>Pseudomonas syringae</i> DC3000	27	8
	PA5083	<i>Pseudomonas aeruginosa</i> PAO1	29	9
Rid3	PSPTO_3006	<i>Pseudomonas syringae</i> DC3000	33	12
	PFL_1385	<i>Pseudomonas fluorescens</i> Pf-5	31	8

^a Rid subfamily assignments were obtained from the PubSEED database (40).

^b Identity (%) to *S. enterica* RidA was calculated from the amino acid sequence alignment given in Fig 1.

^c For each organism, the total number of Rid proteins encoded in the genome is shown.

Table 3.4 Rid proteins deaminate a range of imine substrates^a.

Reaction	Subfamily	Rid protein	Rate of semicarbazone formation				
			Substrate				
			L-Leu	L-Gln	L-Phe	L-Met	L-his
1	-	None	46 ± 0.7	18 ± 0.6	21 ± 0.9	32 ± 0.3	22 ± 2.3
2	RidA	RidA	6 ± 0.4	3 ± 0.2	16 ± 1.3	4 ± 0.3	19 ± 0.1
3	Rid1	PA0814	5 ± 0.8	1 ± 0.3	4 ± 0.7	4 ± 0.4	12 ± 0.3
4		ACIAD3089	9 ± 0.1	4 ± 0.8	14 ± 1.3	6 ± 0.3	18 ± 1.6
5	Rid2	PSPTO_0102	3 ± 0.1	0 ± 0.3	3 ± 0.1	4 ± 0.2	9 ± 0.4
6		PA5083	2 ± 0.4	1 ± 0.4	3 ± 0.9	3 ± 0.2	9 ± 0.3
7	Rid3	PFL_1385	2 ± 0.6	1 ± 0.3	3 ± 0.2	5 ± 0.2	9 ± 0.3
8		PSPTO_3006	2 ± 0.2	0 ± 0.7	3 ± 0.2	7 ± 0.4	8 ± 0.4

^a Each reaction mixture (100 μ L) contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), *Crotalus adamanteus* L-amino acid oxidase (0.4 μ M), bovine liver catalase (24 Units) and the indicated Rid protein (10 μ M). Reactions were initiated with the indicated amino acid substrate to a concentration of 10 mM. The path length of each well was measured and the absorbance at 248 nm was monitored for five minutes. The rate of semicarbazone formation was calculated from the observed rate using the molar extinction coefficient for semicarbazone ($\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$). Values represent the average and standard deviation of three replicates (average $\pm$ SD). The reactions are numbered in the leftmost column.

CHAPTER 4

HETEROLOGOUS EXPRESSION OF PLP-INDEPENDENT RACEMASES CAN REDUCE 2-AMINOACRYLATE ACCUMULATION IN *SALMONELLA ENTERICA*³

³ Hodge-Hanson K. M., A. Zoino, and D. M. Downs. *To be submitted to Journal of bacteriology*

4.1 ABSTRACT

The RidA protein (PF01042) from *Salmonella enterica* is a deaminase that quenches 2-aminoacrylate (2AA), and other reactive metabolites. In the absence of RidA, 2AA accumulates, damages cellular enzymes and compromises the metabolic network. *In vitro*, RidA homologs from all domains of life deaminate 2-aminoacrylate and RidA proteins from plants, bacteria, yeast, and humans complement the mutant phenotypes of a *ridA* mutant strain of *S. enterica*. In the present study a Class I methanogenic archaeon, *Methanococcus maripaludis* S2 was used to probe alternative mechanisms that can restore metabolic balance in the absence of RidA. The *M. maripaludis* ORF MMP0739, annotated as an aspartate/glutamate racemase, complemented a *ridA* mutant strain and reduced the intracellular 2-aminoacrylate burden. The aspartate/glutamate racemase YgeA from *Escherichia coli* and *S. enterica*, when provided *in trans*, similarly restored wild-type growth to a *ridA* mutant. These results uncovered a new mechanism to ameliorate metabolic stress and suggested direct quenching by RidA is not the only means a cell has to quench 2AA.

Importance

The cellular metabolic network includes promiscuous enzymatic activities and reactive metabolites that are controlled to optimize cell growth and fitness. 2-aminoacrylate is an endogenously generated reactive metabolite that can damage cellular enzymes if not directly quenched by the conserved deaminase RidA. This study used an archaeon to identify a RidA-independent mechanism to prevent metabolic stress caused by 2AA. The data suggest a gene product annotated as an aspartate/glutamate racemase (MMP0739) produces a metabolite that can quench 2AA, expanding our understanding of strategies available to quench reactive metabolites.

4.2 INTRODUCTION

The archetypal member of the RidA/YER057c/UK114 protein superfamily, RidA from *Salmonella enterica*, is an imine deaminase with broad specificity (1, 2). Characterization of this protein led to the demonstration that the endogenously generated metabolite 2-aminoacrylate (2AA) causes cellular stress by damaging metabolic enzymes. In *S. enterica*, RidA ameliorates the potential for stress by quenching 2AA (1, 3-6). The Rid family is sequence-diverse and conserved across all domains of life (2), suggesting the *S. enterica* paradigm is widespread. In fact, RidA proteins from all three domains quench 2AA *in vivo* and *in vitro* (1, 5, 7).

Both the generation of 2AA and the consequences of its persistence are linked to the essential cofactor pyridoxal 5'-phosphate (PLP) (1, 5, 8-10). 2AA is a three-carbon enamine produced as a mechanistic intermediate by several pyridoxal 5'-phosphate (PLP)-dependent enzymes, notably serine/threonine dehydratases (1, 11-14). 2AA accumulation leads to decreased activity of PLP-dependent enzymes, including branched-chain amino acid transaminase (EC 2.6.1.42, IlvE) (5, 15), serine hydroxymethyltransferase (EC 2.1.2.1, GlyA) (8, 16), and alanine racemase (EC 5.1.1.1, Alr and DadX) (9) in *S. enterica*. Collectively, inactivation of these enzymes leads to growth defects in *S. enterica ridA* strains (5, 8, 9). Diverse phenotypic defects have been reported in other organisms when the relevant RidA homolog(s) is deleted. Several of these phenotypes, including developmental defects in plants (7, 17) and decreased mitochondrial DNA stability in yeast (18-20) (Ernst, submitted) were associated with the accumulation of 2AA.

Definition of the RidA paradigm in *S. enterica* highlighted the potential for uncontrolled metabolites to cause damage and emphasized key features of the complex metabolic interactions that can occur in the cellular milieu (Figure 1). First, reactive metabolites that are obligatory intermediates in conserved PLP-dependent mechanisms can damage cellular processes and

compromise fitness. Second, the ability of 2AA to cause cellular damage requires that it leaves the active site of the enzyme generator, and survives the cellular milieu long enough to find and damage target enzymes. The latter point implies a dearth of free water in the cellular milieu that has implications in the evolution of metabolic pathway strategies (21, 22). The extensive metabolic diversity of life makes it probable that 2AA is not the only metabolite with the potential to damage cellular components, and furthermore, enzymatic quenching is unlikely to be the only mechanism used to modulate reactive molecules. Identifying other protein(s) or metabolic tactics that can eliminate 2AA stress would provide insights into strategies that have arisen to deal with metabolite stress.

In this study 2AA was used as a model endogenous stressor in a search for non-RidA proteins that could prevent cellular damage. A genomic library of the methanogenic archaeon, *Methanococcus maripaludis* S2, which lacks an annotated RidA in the genome, was used to isolate multicopy suppressors of a *S. enterica ridA* mutant. *In vivo* experiments determined the ORF MMP0739 (a putative aspartate/glutamate racemase) functionally substituted for RidA in *S. enterica*. A working model suggested heterologous complementation was due to quenching of 2AA by a metabolite generated by MMP0739, reflecting a new strategy to deal with metabolite stressors.

4.3 MATERIALS AND METHODS

Bacterial strains, chemicals and media

All strains used in this study are derivatives of *S. enterica* serovar Typhimurium LT2, and are described with relevant plasmids in Table 1. Primers used to construct plasmids are listed in Table 2. Tn10d(Tc) refers to the transposition-deficient mini-Tn10 (23). Difco Nutrient Broth (8 g/ liter) with NaCl (5g/ liter) was used as a rich medium for *S. enterica* and Lysogeny Broth (LB)

was used for *E. coli*. No-carbon E medium (NCE) (24), supplemented with MgSO₄ (1 mM), trace minerals (25), with glucose (11 mM) or pyruvate (50 mM) served as minimal medium. Solid medium contained Difco agar (15 g/ liter). When indicated, supplements were present at the following concentrations unless otherwise indicated: serine, 5 mM; isoleucine, 0.3 mM; cysteine, 250 µM; glycine, 1 mM; kanamycin, 50 mg/ liter; ampicillin, 150 mg/ liter; chloramphenicol, 20 mg/ liter. Kanamycin and Tris were purchased from Amresco (Solon, OH), and all other chemicals were from Sigma Aldrich (St. Louis, MO).

Growth analyses in liquid medium were done in a BioTek ELx808 plate reader in a 96-well microtiter plate. *S. enterica* cultures grown to full density in NB (2 mL) were used to inoculate the indicated medium (2.5% inoculum). Plates were incubated at 37°C with shaking and growth was measured as the change in optical density (OD) at 650 nm. Strains were grown in triplicate and error bars shown indicate the standard error of the mean. Growth curves were plotted and statistical analyses performed using Graph Pad Prism 6.0g.

Genomic library construction

A library containing *Methanococcus maripaludis* was generated using blunt cloning and the commercially available HC-Kan pSMART vector (Lucigen). Genomic DNA (18 µg) was prepared in TE buffer (pH 8.0) containing glycerol (10%), and sheared in 30-second increments using a Nebulizer (Invitrogen) according to the manufacturer's protocol. Following each nebulizer treatment, a sample of DNA (5 µL) was run on a 1% agarose gel. After a total treatment time of 120 seconds, the DNA fragments ranged from 1-3 kb in size. Sheared DNA was isolated via ethanol precipitation and a total of 14 µg was recovered. Blunt ends were repaired using the DNA Terminator End Repair Kit (Lucigen), and DNA was again isolated via ethanol precipitation. Ligation reactions and subsequent transformations into competent cells

were done as described in the CloneSMART Blunt Cloning Kit manual (Lucigen). Briefly, twenty reaction mixtures (10 μ L each) were prepared containing end-repaired DNA (500 ng), CloneSmart Vector Premix and CloneSmart DNA ligase. Reactions were kept at room temperature for 30 minutes. An aliquot of ligation mix (4 μ L) was transformed into electrocompetent *E. coli* cells (25 μ L) and transferred to the recovery medium supplied with the CloneSMART kit. Cells were incubated for 1 hour at 37°C before plating to LB medium containing kanamycin.

Transformations were continued until a minimum of 47,000 clones was obtained. Based on the number of clones isolated from control transformations without insert DNA, we calculated 23,500 clones in the library should contain a fragment of *M. maripaludis* DNA. The *M. maripaludis* genome is 1.7 Mb in size, and contains 1,722 protein-encoding genes (26), therefore the library achieved 14x coverage. Batches of 1,000-2,000 colonies were pooled in LB, aliquotted into cryovials containing DMSO, and stored at -80°C. Scrapings from each vial were used to inoculate LB (5 mL) containing kanamycin and plasmid DNA was isolated from these cultures using E.Z.N.A. plasmid midi kits (Omega, Norcross, GA).

Sub-cloning the MMP0739 codon optimized allele

An MMP0739 allele was codon-optimized for expression in *Escherichia coli*, synthesized, and cloned into pET28a at NheI and EcoRI restriction sites by Genscript (Piscataway, NJ). The resulting plasmid was designated pDM1516 as used as template in PCR. The cloned portion of DNA was amplified using primers PR844 and PR846, which included amplification of the stop codon and introduced BspQI cut sites but did not include any his₆ or T7 tags. A digested PCR product was ligated into a pBAD24 vector modified to include a BspQI cut site (pCV1) as

previously described (27). The resulting plasmid was named pDM1520, and the insert was confirmed by sequencing (Eton BioScience Inc., San Diego, CA).

Construction of site-directed mutations

Site-directed mutagenic primers were designed using the Agilent Quickchange Primer Design online tool. Amplification reaction mixtures (50 μ L) contained template DNA (100 ng), primers (250 nM), dNTP's (250 nM), DMSO (1 μ L), Pfu Ultra reaction buffer (5 μ L) and Pfu Ultra II polymerase (1 μ L). An extension time of 2.5 minutes per kilobase of the entire plasmid was used. A gradient spanning ± 5 $^{\circ}$ C of the estimated melting temperature was done for each primer pair, and the samples were pooled before digesting with DpnI (1 μ L) for 8 hours. The digested sample (5 μ L) was transformed into electrocompetent *E. coli* DH5-alpha cells. Plasmid DNA was harvested from colonies growing on LB containing the appropriate drug, and mutations were confirmed by sequence analysis performed by Eton Bioscience (San Diego, CA).

Isoleucine transaminase activity assay

Isoleucine transaminase activity was measured using permeabilized cells as previously described (15). Freshly revived strains were used to start overnight cultures in order for optimal growth of strains harboring constructs with active racemases. Cultures were grown in minimal NCE medium (5 mL) containing ampicillin (15 mg/ liter) and arabinose (0.2%) with glycerol (30 mM) as the sole carbon source. When cultures reached full density, cells were pelleted, washed in NCE medium, and pellets were kept at -20 $^{\circ}$ C until assayed. Cell pellets were resuspended in potassium phosphate buffer (250 μ L, pH 7.5). Reaction mixtures (180 μ L) contained cell resuspension (50 μ L), PLP (50 μ M), Popculture Reagent (20 μ L), and α -ketoglutarate (10 mM). Mixtures were incubated at 37 $^{\circ}$ C for 10 minutes prior to the addition of 0.2 M isoleucine (20 μ L). After 20 minutes, reactions were quenched with the addition of 2,4-dinitrophenylhydrazine

(DNPH, 200 μ L) to derivatize the product 2-ketomethylvalerate (2-KMV). Samples were incubated for 2 minutes at 37 °C, subject to organic extraction with toluene (1 mL), and washed with 0.5 N HCl. An aliquot (700 μ L) of the top layer was mixed with 1.5 N NaOH to promote chromophore formation. 200 μ L of the aqueous layer containing the chromophore was removed and the absorbance at 540 nm was measured using a SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA). Chromophore concentration was determined via comparison to a standard curve of 2-KMV extractions. An aliquot (25 μ L) of resuspended cells was lysed with PopCulture Reagent and the total protein content was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Activity is given in nmol 2-KMV produced per minute per milligram of total cellular protein.

4.4 RESULTS AND DISCUSSION

***Methanococcus maripaludis* gene product MMP0739 complements a *S. enterica ridA* mutant**

S. enterica strains lacking *ridA* are unable to grow on minimal glucose medium supplemented with serine (5 mM) or cysteine (250 μ M) due to the damage caused by 2-aminoacrylate (2AA) accumulation (5, 8, 9, 28). Growth can be restored by expressing a functional *ridA* gene *in trans*, or by supplementing the media with glycine or isoleucine. The genome of the methanogenic archaeon *M. maripaludis* lacks any identifiable Rid proteins. A high copy plasmid library of *M. maripaludis* genomic DNA was used to transform a *S. enterica ridA* strain (DM12920). Kanamycin-resistant transformants were selected on nutrient medium and replica printed to minimal glucose medium containing serine. Colonies from the serine plates were streaked for isolation and growth was assessed on both medium with serine and medium with cysteine. Of more than 47,000 kanamycin-resistant transformants, seven grew in the presence of either serine or cysteine, suggesting the plasmids complemented the *ridA* defect.

Plasmids from each of the seven transformants were isolated and reintroduced into the parent strain. After confirming the plasmids restored growth of a *S. enterica ridA* strain, the plasmid inserts were sequenced.

The sequence of the seven plasmids identified unique inserts that overlapped with a single ORF, MMP0739 (Figure 2). The plasmid with the smallest insert was designated pDM1438 and used for further characterization. The impact of MMP0739 on the growth of a *ridA* mutant was evaluated and some of the results are shown in Figure 3. Several points were taken from these data. First, the pDM1438 plasmid restored growth to a *ridA* mutant in medium with either serine or cysteine. Exogenous isoleucine or glycine restored growth in both conditions, consistent with the previously defined role of 2AA in the growth defect. The other six plasmids similarly restored growth of the *ridA* strain, suggesting the MMP0739 ORF was responsible for the effect (data not shown). Two consecutive stop codons (UAA, UAA) were introduced into the coding sequence of MMP0739, at residues D28 and K29. The resulting plasmid (pDM1445) failed to complement the *ridA* mutant, indicating translation of the MMP0739 ORF was required for suppression (Figure 3). The pSMART plasmid used to make the library has no defined promoter (29), so the expression of MMP0739 was presumed to be directed by the insert DNA. Consistent with this scenario, there was a 40 bp DNA region upstream of MMP0739 that encoded putative promoter elements including an AT-rich sequence at the -23 to -30 position that may include a TATA box and transcription factor B recognition element (BRE) (Figure 2) (30, 31). Together, these latter data supported the conclusion that MMP0739 was transcribed from its native promoter and translation of the MMP0739 gene product was required to functionally replace RidA in *S. enterica*.

Putative aspartate/glutamate racemases substitute for *RidA* *in vivo*

The MMP0739 gene product was annotated as a member of the aspartate/glutamate racemase superfamily (26). In contrast to many racemases, these aspartate/glutamate racemases are PLP-independent enzymes and catalyze bidirectional racemization using a “two-base” mechanism that typically involves two active site cysteinyl residues (32-36). In this mechanism, the pair of cysteines deprotonate and re-protonate the substrate at opposite faces to produce both stereoisomers (Figure 4.) Enzymes of this class from *Pyrococcus horikoshii* (37), *Desulfurococcus* (38), *Bifidobacterium bifidum* (39), or *S. thermophilus* (32) have been characterized *in vitro*. *E. coli* contains two PLP-independent racemases. MurI, a reversible glutamate racemase involved in peptidoglycan biosynthesis (33, 40) is a member of this family and is 19% identical to MMP0739. MurI has been extensively characterized *in vitro* and *in vivo* (34, 41). *E. coli* YgeA is also a member of this family and by one report, has racemase activity with aspartate and glutamate (42). *S. enterica* contains MurI and YgeA, as well as two ORFs annotated as putative PLP-independent mandelate racemases (STM3697 and STM3833).

Proteins from *E. coli* and *S. enterica* were tested to determine if multicopy suppression of *ridA* mutant phenotypes was a common characteristic of the PLP-independent racemase family that included MMP0739. Plasmids from the ASKA collection of pCA24N IPTG-inducible vectors (43) harboring *Escherichia coli* *murI* or *ygeA* were introduced into a *ridA* mutant strain of *S. enterica* (DM12920), and growth of the resulting strains was assessed. Plasmid pCA24N-*ygeA*, but not plasmid pCA24N-*murI*, restored growth of a *ridA* strain in the presence of serine or cysteine when expression of the gene was induced (Figure 5). Control experiments confirmed that plasmid pCA24N-*murI* encoded a functional protein, because it complemented the D-glutamate auxotrophy of *E. coli* strain WM335 (*murI*; data not shown) (44, 45). Neither plasmid

pDM1438 nor pCA24N-*ygeA* complemented the defect of WM335, showing neither MMP0739 nor YgeA had significant L- to D-glutamate racemase activity. The phenotypes of *S. enterica* *ridA* mutants show that *ygeA* does not substitute for RidA natively. Although it was formally possible that a racemase product mediated the complementation, this scenario was eliminated when exogenous supplementation with glutamate/aspartate stereoisomers failed to stimulate growth of a *ridA* mutant strain.

Presumed active site residues are required for suppression

YgeA and MMP0739 differ from the more characterized members of the PLP-independent racemase family in that they contain a single conserved cysteine residue in the presumed active site (Figure 6). In these two proteins, a threonine replaces the second cysteine, a substitution that has implications for the catalytic mechanism. *E. coli* variants YgeA^{C197S} and YgeA^{T83C} had no aspartate racemase activity *in vitro* (42). The requirement for these residues in substituting for RidA function was addressed with targeted mutagenesis. *S. enterica* YgeA is 80% identical to that of *E. coli*, and variants of this protein were constructed and tested *in vivo* in complementation assays. Unlike wild-type *ygeA*, neither variant supported growth of a *ridA* mutant strain in the presence of serine or cysteine (data not shown). MMP0739 has high identity to YgeA surrounding the active site and plasmids that encoded the MMP0739^{C197A} or MMP0739^{T83C} variants similarly failed to complement a *ridA* mutant strain (Figure 7). Unexpectedly, substituting Thr83 with alanine produced different results in MMP0739 and YgeA. Expression of the MMP0739^{T83A} variant rescued growth of a *ridA* strain in medium containing serine or cysteine, while expression of the YgeA^{T83A} variant failed to complement the *ridA* mutant.

MMP0739 and YgeA decrease 2AA load in a *ridA* mutant

In the absence of RidA, 2AA accumulates and targets PLP-dependent enzymes, including transaminase IlvE in *S. enterica*. Because 2AA is unstable and cannot be directly measured, the specific activity of IlvE is used as a proxy for the 2AA burden in a strain (5). Plasmid constructs that carry *ridA*, MMP0739 or *ygeA*, were transformed into a *ridA* mutant strain. Activity of IlvE was assayed in crude extracts of each of the resulting strains (Figure 8). When expression was induced, RidA, MMP0739 and YgeA improved IlvE activity in the respective strains. Furthermore, expression of the MMP0739^{T83C} and MMP073^{C197A} variant proteins failed to improve IlvE activity, while MMP0739^{T83A} had an intermediary and statistically significant effect (Figure 8). These results generally correlated with the complementation data, and were consistent with a mechanism of suppression in which activity of MMP0739 and YgeA quenched 2AA.

Working model for multicopy suppression by MMP0739

Members of the PLP-independent racemase family have a catalytic mechanism that requires a dual cysteine active site (Figure 4). The two members of this family that substitute for RidA have a cysteine-threonine configuration, and each of these residues impacts their ability to complement a *ridA* mutant *in vivo*. We hypothesize that a catalytic intermediate of the racemase mechanism is involved in quenching 2AA. Further, we suggest that lack of the second cysteine affects the persistence of this intermediate, allowing it to interact with 2AA that accumulates in a *ridA* mutant (Figure 9). Thr83 is not expected to initiate the reaction and the mechanism is unidirectional. A cysteine residue at this position allows a reversible reaction, as described for the PLP-independent racemase mechanism (32-35). The complementation and IlvE activity data presented above are consistent with this scenario and emphasize the potential for metabolite

quenching as a cellular approach to controlling endogenous stress. Efforts to assay MMP0739 or YgeA *in vitro* for RidA-like enamine deaminase activity failed to detect activity.

Conclusions

The growth defect of a *ridA* strain of *S. enterica* is caused by the accumulation of 2AA that leads to the collective inactivation of PLP-dependent enzymes. There are two general ways to bypass the growth defect of a *ridA* mutant, i) reduce the accumulation of 2AA, or ii) absorb the defect by altering flux through the metabolic network (10). The latter allows growth without decreasing the 2AA load in the cell. This study showed that expression of two members of an aspartate/glutamate racemase family with a cysteine/threonine catalytic pair (i.e. MMP0739, YgeA) can reduce the 2AA burden of a cell. While the current data do not differentiate between a direct or indirect role for these proteins in reducing 2AA, we favor a mechanism in which the carbanion of an intermediate generated by the putative racemase reacts with 2AA either on the enzyme or in solution to form a stable molecule (Figure 9). Such a scenario could involve the predicted (aspartate/glutamate), or non-canonical substrate of these enzymes. Significantly, these possibilities are not trivial to address with *in vitro* approaches, given the short half-life of 2AA and the absence of YgeA or MMP0739 activity *in vitro*. Efforts that characterized the RidA paradigm emphasized that *in vitro* conditions can be misleading about the cellular milieu in the case of low-level, reactive metabolites due to the use of aqueous solutions (1). Emerging technologies in metabolomics, when pursued in the context of genetic and phenotypic analysis, will facilitate our increased understanding of the subtleties of metabolism and the strategies that have evolved to control metabolic stress. Current methods to detect 2AA damage rely on monitoring one of the four known damaged enzymes, and developing sensitive technologies to quantify or track 2AA in an untargeted manner *in vivo* will be valuable ventures. In summary,

the results herein suggest that cells can control reactive metabolites not only the Rid paradigm of enzymatic quenching, but also by production of molecules that themselves will quench rogue metabolites.

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4.6 REFERENCES

1. Lambrecht JA, Flynn JM, Downs DM. Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions. *The Journal of biological chemistry*. 2012;287(5):3454-61.
2. Niehaus TD, Gerdes S, Hodge-Hanson K, Zhukov A, Cooper AJ, ElBadawi-Sidhu M, et al. Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC genomics*. 2015;16:382.
3. Enos-Berlage JL, Langendorf MJ, Downs DM. Complex metabolic phenotypes caused by a mutation in *yjgF*, encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of bacteriology*. 1998;180(24):6519-28.
4. Christopherson MR, Schmitz GE, Downs DM. YjgF is required for isoleucine biosynthesis when *Salmonella enterica* is grown on pyruvate medium. *Journal of bacteriology*. 2008;190(8):3057-62.
5. Lambrecht JA, Schmitz GE, Downs DM. RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life. *mBio*. 2013;4(1):e00033-13.
6. Downs DM, Ernst DC. From microbiology to cancer biology: the Rid protein family prevents cellular damage caused by endogenously generated reactive nitrogen species. *Molecular microbiology*. 2015;96(2):211-9.
7. Niehaus TD, Nguyen TND, Gidda SK, ElBadawi-Sidhu M, Lambrecht JA, McCarty DR, et al. Arabidopsis and Maize RidA Proteins Preempt Reactive Enamine/Imine Damage to

- Branched-Chain Amino Acid Biosynthesis in Plastids. *The Plant Cell*. 2014;26(7):3010-22.
8. Flynn JM, Christopherson MR, Downs DM. Decreased coenzyme A levels in *ridA* mutant strains of *Salmonella enterica* result from inactivated serine hydroxymethyltransferase. *Molecular microbiology*. 2013;89(4):751-9.
 9. Flynn JM, Downs DM. In the absence of *RidA*, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5'-phosphate cofactor. *Journal of bacteriology*. 2013;195(16):3603-9.
 10. Ernst DC, Downs DM, Metcalf WW. 2-Aminoacrylate Stress Induces a Context-Dependent Glycine Requirement in *ridA* strains of *Salmonella enterica*. *Journal of Bacteriology*. 2016;198(3):536-43.
 11. Schnackerz KD, Ehrlich JH, Giesemann W, Reed TA. Mechanism of action of D-serine dehydratase. Identification of a transient intermediate. *Biochemistry*. 1979;18(16):3557-63.
 12. Datta P, Bhadra R. Biodegradative threonine dehydratase. Reduction of ferricyanide by an intermediate of the enzyme-catalyzed reaction. *European journal of biochemistry*. 1978;91(2):527-32.
 13. Phillips AT, Wood WA. The mechanism of action of 5'-adenylic acid-activated threonine dehydrase. *The Journal of biological chemistry*. 1965;240(12):4703-9.
 14. Chargaff E, Sprinson DB. Studies on the mechanism of deamination of serine and threonine in biological systems. *Journal of Biological Chemistry*. 1943;151(1):273-80.
 15. Schmitz G, Downs DM. 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. *Journal of bacteriology*. 2004;186(3):803-10.
 16. Ernst DC, Anderson ME, Downs DM. L-2,3-diaminopropionate generates diverse metabolic stresses in *Salmonella enterica*. *Mol Microbiol*. 2016;101(2):210-23.
 17. Leitner-Dagan Y, Ovadis M, Zuker A, Shklarman E, Ohad I, Tzfira T, et al. CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. *Planta*. 2006;225(1):89-102.
 18. Oxelmark EM, Antonio; Malanchi, Ilaria; Magherini, Francesca; Jaquet, Laurence; Hajibagheri, M. A. Nasser; Blight, Kenneth J.; Jauniaux, Jean-Claude; Tommassino, Massimo. Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome. *Molecular and cellular biology*. 2000;20(20):7784-97.

19. Kim J-M, Yoshikawa H, Shirahige K. A member of the YER057c/yjgf/Uk114 family links isoleucine biosynthesis to intact mitochondria maintenance in *Saccharomyces cerevisiae*. *Genes to cells*. 2001;6:507-17.
20. Accardi R, Oxelmark E, Jauniaux N, de Pinto V, Marchini A, Tommasino M. High levels of the mitochondrial large ribosomal subunit protein 40 prevent loss of mitochondrial DNA in null *mmf1* *Saccharomyces cerevisiae* cells. *Yeast*. 2004;21(7):539-48.
21. Kreuzer-Martin HW, Lott MJ, Ehleringer JR, Hegg EL. Metabolic processes account for the majority of the intracellular water in log-phase *Escherichia coli* cells as revealed by hydrogen isotopes. *Biochemistry*. 2006;45(45):13622-30.
22. Christopherson MR, Lambrecht JA, Downs D, Downs DM. Suppressor analyses identify threonine as a modulator of *ridA* mutant phenotypes in *Salmonella enterica*. *PloS one*. 2012;7(8):e43082.
23. Way JC, Davis MA, Morisato D, Roberts DE, Kleckner N. New Tn10 derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene*. 1984;32(3):369-79.
24. Vogel HJ, Bonner DM. Acetylornithinase of *Escherichia coli*: partial purification and some properties. *The Journal of biological chemistry*. 1956;218(1):97-106.
25. Balch WE, Wolfe RS. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Applied and environmental microbiology*. 1976;32(6):781-91.
26. Hendrickson EL, Kaul R, Zhou Y, Bovee D, Chapman P, Chung J, et al. Complete genome sequence of the genetically tractable hydrogenotrophic methanogen *Methanococcus maripaludis*. *Journal of bacteriology*. 2004;186(20):6956-69.
27. VanDrisse CM, Escalante-Semerena JC. New high-cloning-efficiency vectors for complementation studies and recombinant protein overproduction in *Escherichia coli* and *Salmonella enterica*. *Plasmid*. 2016;86:1-6.
28. Ernst DC, Lambrecht JA, Schomer RA, Downs DM. Endogenous Synthesis of 2-Aminoacrylate Contributes to Cysteine Sensitivity in *Salmonella enterica*. *Journal of Bacteriology*. 2014;196(18):3335-42.
29. McFarland KN, Liu J, Landrian I, Godiska R, Shanker S, Yu F, et al. SMRT Sequencing of Long Tandem Nucleotide Repeats in SCA10 Reveals Unique Insight of Repeat Expansion Structure. *PloS one*. 2015;10(8):e0135906.
30. Pu YG, Jiang YL, Ye XD, Ma XX, Guo PC, Lian FM, et al. Crystal structures and putative interface of *Saccharomyces cerevisiae* mitochondrial matrix proteins Mmf1 and Mam33. *J Struct Biol*. 2011;175(3):469-74.

31. Zhang J, Li E, Olsen GJ. Protein-coding gene promoters in *Methanocaldococcus* (*Methanococcus*) *jannaschii*. *Nucleic acids research*. 2009;37(11):3588-601.
32. Yamauchi T, Choi SY, Okada H, Yohda M, Kumagai H, Esaki N, et al. Properties of aspartate racemase, a pyridoxal 5'-phosphate-independent amino acid racemase. *The Journal of biological chemistry*. 1992;267(26):18361-4.
33. Glavas S, Tanner ME. Active site residues of glutamate racemase. *Biochemistry*. 2001;40(21):6199-204.
34. Yoshimura T, Esak N. Amino acid racemases: functions and mechanisms. *Journal of bioscience and bioengineering*. 2003;96(2):103-9.
35. Ohtaki A, Nakano Y, Iizuka R, Arakawa T, Yamada K, Odaka M, et al. Structure of aspartate racemase complexed with a dual substrate analogue, citric acid, and implications for the reaction mechanism. *Proteins*. 2008;70(4):1167-74.
36. Liu X, Gao F, Ma Y, Liu S, Cui Y, Yuan Z, et al. Crystal structure and molecular mechanism of an aspartate/glutamate racemase from *Escherichia coli* O157. *FEBS letters*. 2016;590(8):1262-9.
37. Liu L, Iwata K, Kita A, Kawarabayasi Y, Yohda M, Miki K. Crystal structure of aspartate racemase from *Pyrococcus horikoshii* OT3 and its implications for molecular mechanism of PLP-independent racemization. *Journal of molecular biology*. 2002;319(2):479-89.
38. Yohda M, Endo I, Abe Y, Ohta T, Iida T, Maruyama T, et al. Gene for aspartate racemase from the sulfur-dependent hyperthermophilic archaeum, *Desulfurococcus* strain SY. *The Journal of biological chemistry*. 1996;271(36):22017-21.
39. Yamashita T, Ashiuchi M, Ohnishi K, Kato S, Nagata S, Misono H. Molecular identification of monomeric aspartate racemase from *Bifidobacterium bifidum*. *European journal of biochemistry*. 2004;271(23-24):4798-803.
40. Doublet P, van Heijenoort J, Bohin JP, Mengin-Lecreulx D. The *murI* gene of *Escherichia coli* is an essential gene that encodes a glutamate racemase activity. *Journal of bacteriology*. 1993;175(10):2970-9.
41. Fisher SL. Glutamate racemase as a target for drug discovery. *Microbial biotechnology*. 2008;1(5):345-60.
42. Ahn JW, Chang JH, Kim KJ. Structural basis for an atypical active site of an L-aspartate/glutamate-specific racemase from *Escherichia coli*. *FEBS letters*. 2015;589(24 Pt B):3842-7.
43. Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, et al. Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA research : an*

international journal for rapid publication of reports on genes and genomes.
2005;12(5):291-9.

44. Lugtenberg EJJ, Wijsman HJW, Van Zaane D. Properties of a D-Glutamic Acid Requiring Mutant of *Escherichia coli*. Journal of bacteriology. 1973;114(2):499-506.
45. Doublet P, van Heijenoort J, Mengin-Lecreulx D. Identification of the *Escherichia coli* *murI* gene, which is required for the biosynthesis of D-glutamic acid, a specific component of bacterial peptidoglycan. Journal of bacteriology. 1992;174(18):5772-9.
46. Lehninger AL, Nelson DL, Cox MM. Lehninger principles of biochemistry. 3rd ed. New York: Worth Publishers; 2000.
47. Caspi R, Billington R, Ferrer L, Foerster H, Fulcher CA, Keseler IM, et al. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic acids research. 2016;44(D1):D471-80.
48. Yohda M, Okada H, Kumagai H. Molecular cloning and nucleotide sequencing of the aspartate racemase gene from lactic acid bacteria *Streptococcus thermophilus*. Biochimica et biophysica acta. 1991;1089(2):234-40.

Table 4.1 Strains and plasmids

Strain	Relevant Genotype ^a		Description	Derivative of
<i>S. enterica</i> strains ^b				
DM12920	<i>ridA1::Tn10d</i> (Tc)			
DM14941	<i>ridA1::Tn10d</i> (Tc)	pDM1438	<i>M. maripaludis</i> DNA fragment	pSMART HC-Kan
DM14966	<i>ridA1::Tn10d</i> (Tc)	pDM1445	UAA UAA/stop codon	pSMART HC-Kan
DM15396	<i>ridA1::Tn10d</i> (Tc)	pCA24N- <i>ygeA</i> (- <i>gfp</i>)	<i>E. coli ygeA</i>	ASKA collection (43)
DM15397	<i>ridA1::Tn10d</i> (Tc)	pCA24N	Empty vector	ASKA collection (43)
DM15398	<i>ridA1::Tn10d</i> (Tc)	pCA24N- <i>murI</i> (- <i>gfp</i>)	<i>E. coli murI</i>	ASKA collection (43)
DM14846	<i>ridA1::Tn10d</i> (Tc)	pDM1439	<i>S. enterica ridA</i>	pBAD24
DM14847	<i>ridA1::Tn10d</i> (Tc)		Empty vector	pBAD24
DM15862	<i>ridA1::Tn10d</i> (Tc)	pDM1528	MMP0739 (codon-optimized) T83A	pBAD24
DM15780	<i>ridA1::Tn10d</i> (Tc)	pDM1520	MMP0739 (codon-optimized)	pBAD24
DM15863	<i>ridA1::Tn10d</i> (Tc)	pDM1529	MMP0739 (codon optimized) C197A	pBAD24
DM15865	<i>ridA1::Tn10d</i> (Tc)	pDM1531	MMP0739 (codon optimized) T83C	pBAD24
DM15483	<i>ridA1::Tn10d</i> (Tc)	pDM1475	<i>S. enterica ygeA</i>	pBAD24
DM15932	<i>ridA1::Tn10d</i> (Tc)	pDM1493	<i>S. enterica ygeA601</i> (T83C)	pBAD24
DM15933	<i>ridA1::Tn10d</i> (Tc)	pDM1494	<i>S. enterica ygeA602</i> T83A	pBAD24
DM15934	<i>ridA1::Tn10d</i> (Tc)	pDM1495	<i>S. enterica ygeA603</i> C197A	pBAD24
Plasmid		Description		
pSMART HC-Kan		High-copy, blunt-cloning vector, <i>kan</i> ⁺ (Lucigen)		
pBAD24 (pCV1)		P _{araBAD} expression vector, <i>bla</i> ⁺ (27)		

^a Tn10d(Tc) refers to the transposition-defective mini-Tn10(Tn10Δ16Δ17)

^b *Salmonella enterica* strains are derivatives of *Salmonella typhimurium* serovar LT2

Table 4.2 Primers

PR#	Name	Sequence
	SL1	CAGTCCAGTTACGCTGGAGTC (Lucigen)
	SR2	GGTCAGGTATGATTTAAATGGTCAGT (Lucigen)
	pBAD_F	ATGCCATAGCATTTTTATCC (Eton Biosciences)
	pBAD_R	GATTTAATCTGTATCAGG (Eton Biosciences)
		TTTTGCTGAATGGGGAGCTCCAAGTTATTATCTAATCGATTGATTTA
PR309	MMP0739 stop F	TAATTCGATAGT
		ACTATCGAATTATAAATCAATCGATTAGATAATAACTTGGAGCTCCC
PR310	MMP0739 stop R	CATTCAGCAAAA
PR617	ygeA for pBAD24 F	NNGCTCTTCNTTCATGGTGAAAACGATCGGACTGTTG
PR618	ygeA for pBAD24 R	NNGCTCTTCNTTATTATTCTGGGGCCGATGA
PR696	YgeA Thr83Ala 5'	GCATGGTGTTGGCACACAGCACAATGCCTTCTGCG
PR697	YgeA Thr83Ala 3'	CGCAGAAGGCATTGTGCTGTGTGCCAACACCATGC
PR698	YgeA Thr83Cys 5'	CGATTTTATGCATGGTGTTGCAACACAGCACAATGCCTTCTG
PR699	YgeA Thr83Cys 3'	CAGAAGGCATTGTGCTGTGTTGCAACACCATGCATAAAATCG
PR700	YgeA Cys197Ala 5'	CGCAGGGCGTTATTTTTGGCGCTACTGAAATAGGTTTGCTTG
PR701	YgeA Cys197Ala 3'	CAAGCAAACCTATTTTCAGTAGCGCCAAAAATAACGCCCTGCG
PR884	MMP0739 CO pBAD F	NNGCTCTTCNTTCATGAAAACCATCGGTCTGATCG
PR886	MMP0739 CO pBAD R	NNGCTCTTCNTTATCACAGTTTGCTATCTTTCAGAACG
PR901	MMP0739 CO T83A F	GTGTGCATGGTGTTGGCGCAAATCAGGATGCCT
PR902	MMP0739 CO T83A R	AGGCATCCTGATTTGCGCCAACACCATGCACAC
PR903	MMP0739 CO T83C 5'	CGGTGTGCATGGTGTTGCAGCAAATCAGGATGCCTT
PR904	MMP0739 CO T83C 3'	AAGGCATCCTGATTTGCTGCAACACCATGCACACCG
PR905	MMP0739 CO C197A 5'	GCATCATTCTGGGCGCCACCGAGATCCCCG
PR906	MMP0739 CO C197A 3'	GCGGGATCTCGGTGGCGCCCAGAATGATGC

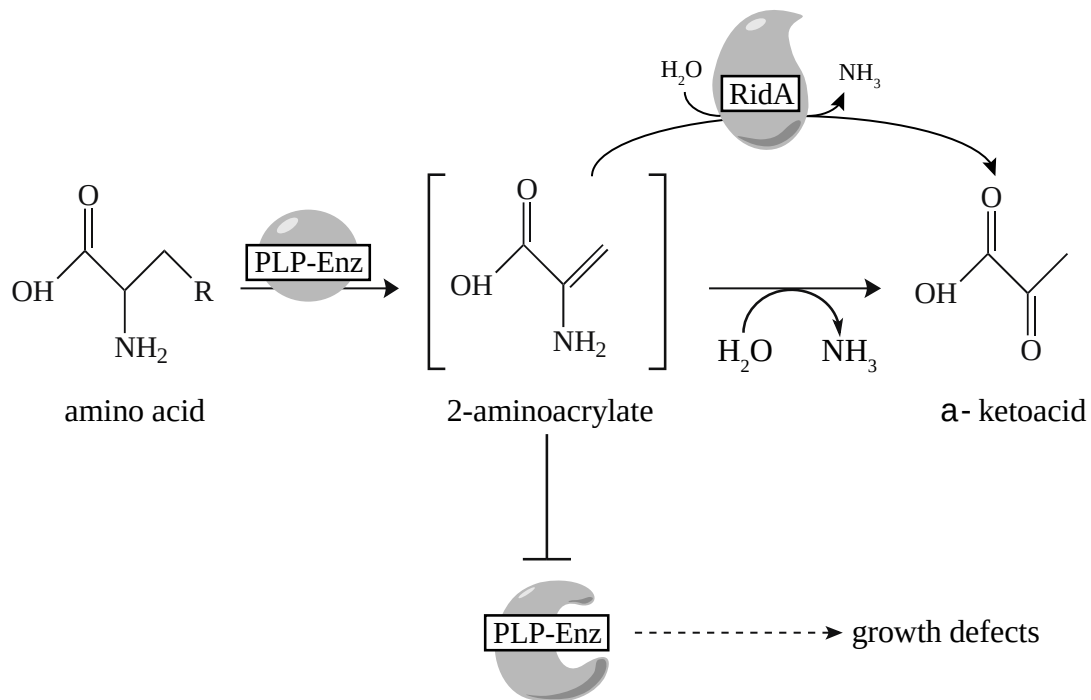


Figure 4.1 The impact of 2AA on the *S. enterica* metabolic network. In the absence of RidA in *S. enterica*, the PLP-dependent generation of 2-aminocrylate in certain β -eliminase reactions can lead inactivation of various PLP-dependent enzymes that catalyzed a different set of reactions. RidA facilitates 2AA hydrolysis, directly quenching the intermediate and forming an α -ketoacid.

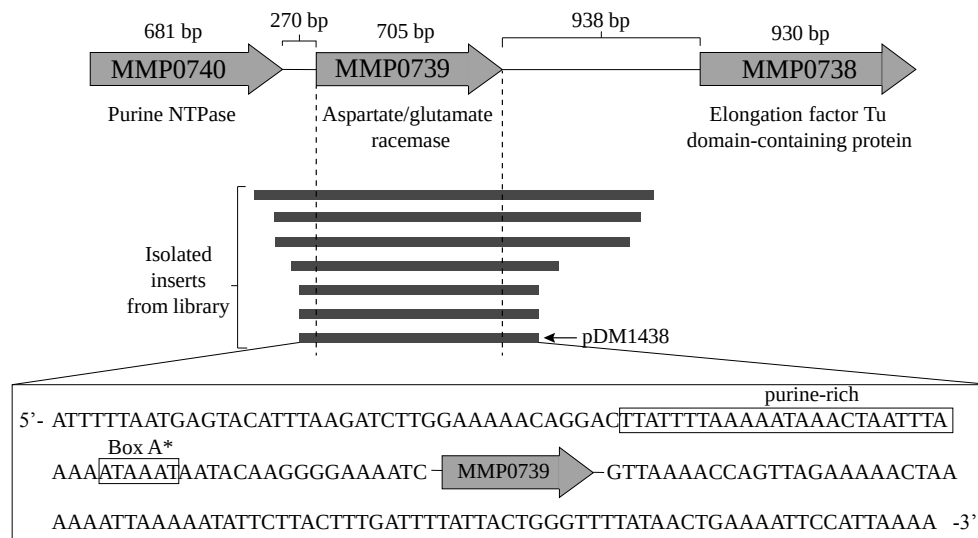


Figure 4.2 MMP0739 is a putative aspartate/glutamate racemase. A region of the *M. maripaludis* chromosome is shown. Annotations are based on the information provided in the Biocyc Database (47). Gene sizes are labeled and intergenic distances are indicated with brackets. The relative sizes of the isolated plasmid inserts are indicated with dark grey lines. The smallest insert was from pDM1438, indicated with an arrow, and the nucleotide sequence is given. Putative promoter elements including a purine-rich region and Box A are boxed.

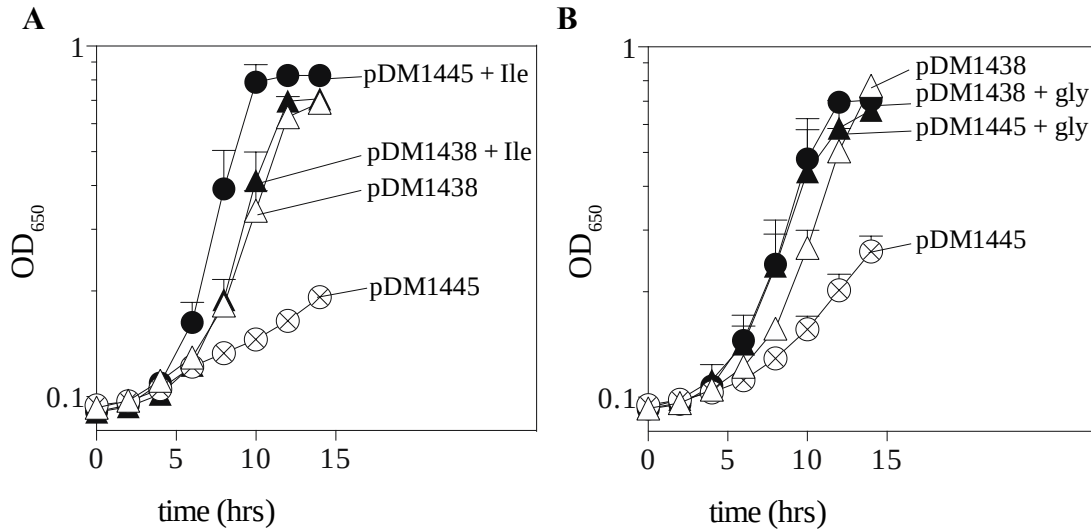


Figure 4.3 MMP0739 high copy plasmid rescues the growth defects of a *ridA* strain. Strains lacking *ridA* (DM12920) were transformed with a pSMART-MMP0739 construct that encoded either wild type (pDM1438, triangles) or MMP0739^{D28stopK29stop} protein (pDM1445, circles). Cultures were grown in minimal glucose medium supplemented with **(A)** 5 mM serine or **(B)** 250 μ M cysteine. Open symbols represent growth without any additional supplementation, and closed symbols represent growth with either isoleucine or glycine added. Growth is reported as optical density at 650 nm (OD₆₅₀) over time. Error bars indicate standard error of the mean for three biological replicates.

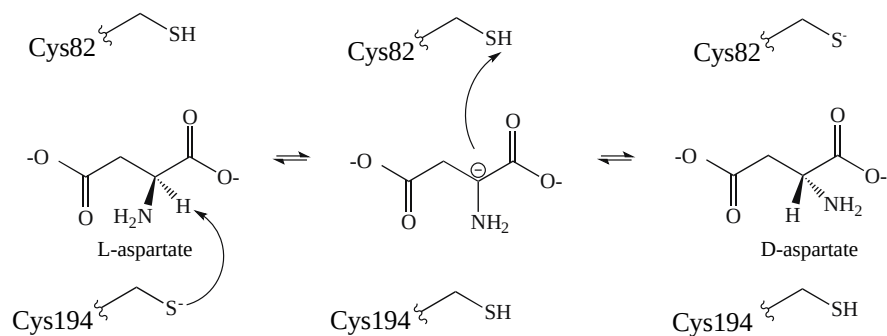


Figure 4.4 PLP-independent racemase mechanism and protein alignment. The bidirectional PLP-independent racemase mechanism is shown with substrate L-aspartate. The active site contains two cysteinyl residues that catalyze bidirectional conversion of aspartate and glutamate stereoisomers (32-35). Catalytic residues are numbered with respect to the *P. horikoshii* aspartate racemase AspR (35).

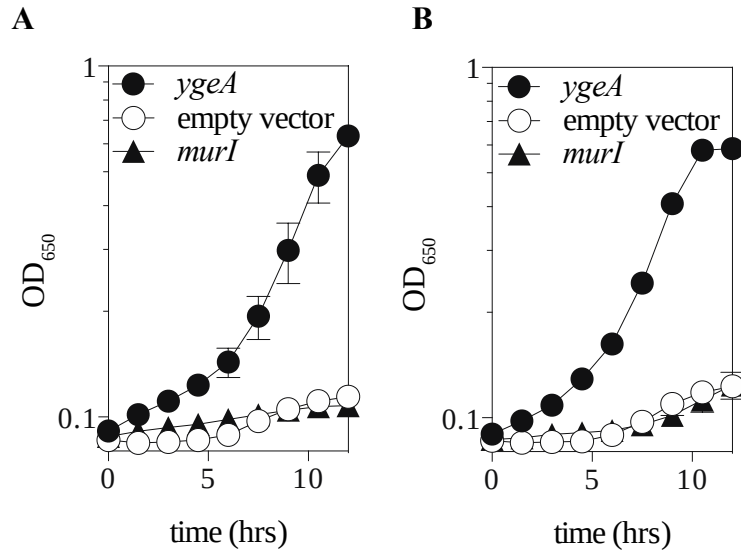


Figure 4.5 *ygeA* complements a *ridA* mutant strain. Strains lacking *ridA* were transformed with pCA24N plasmids harboring *E. coli ygeA*, *murI*, or no insert (empty vector). Cultures were grown in minimal glucose medium containing 0.5 mM IPTG and **(A)** 5 mM serine or **(B)** 250 μ M cysteine. Error bars indicate standard error for three biological replicates.

				*				*	
<i>Ec</i>	MurI	91	A	C	N	T	A	S	202
<i>Se</i>	YgeA	82	C	T	N	T	M	H	195
<i>Mm</i>	MMP0739	82	C	T	N	T	M	H	195
<i>Ph</i>	AspR	81	P	C	N	T	A	H	192
<i>Bb</i>	Mar	84	T	C	N	T	A	H	202
<i>Ef</i>	P29079	83	T	C	N	T	A	H	195

Figure 4.6 Sequence alignment of PLP-independent racemase family proteins. Regions highlighting the PLP-independent cysteinyl residues are shown from an amino acid sequence alignment with *E. coli* MurI (accession no. P22634) *S. enterica* YgeA (accession no. Q8ZMA1), *M. maripaludis* MMP0739 (accession no. Q6LZ90), *P. horikoshii* aspartate racemase (AspR; accession no. PH0670) (35), *Bifidobacterium bifidum* monomeric aspartate racemase (Mar; accession no. AB179841) (39), and *Enterococcus faecium* aspartate racemase (accession no. P29079) (48). The asterisks mark the position of the two conserved cysteinyl residues in the current model for the catalytic mechanism.

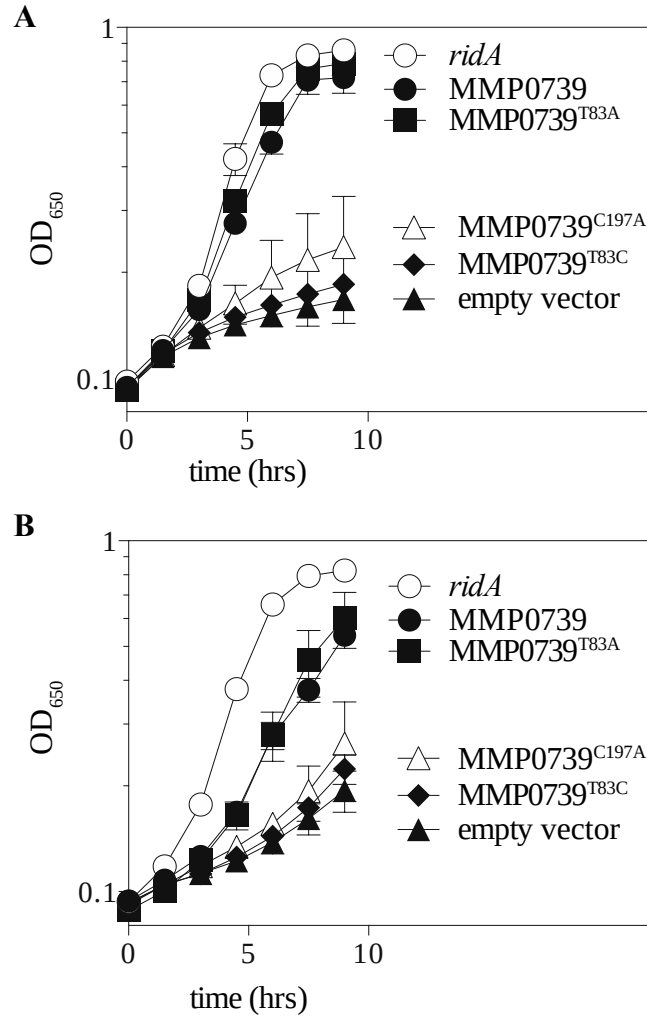


Figure 4.7 MMP0739 mutant analysis in *S. enterica ridA* strain. A strain lacking *ridA* (DM12920) was transformed with pBAD24 plasmids harboring *M. maripaludis* MMP0739, *S. enterica ridA*, or mutant MMP0739 allele encoding T83A, T83C, or C197A variant proteins, or no insert (empty vector). Growth was assessed in minimal glucose medium containing 0.2% arabinose and **(A)** 5 mM serine or **(B)** 250 μM cysteine. Error bars represent standard error for three biological replicates.

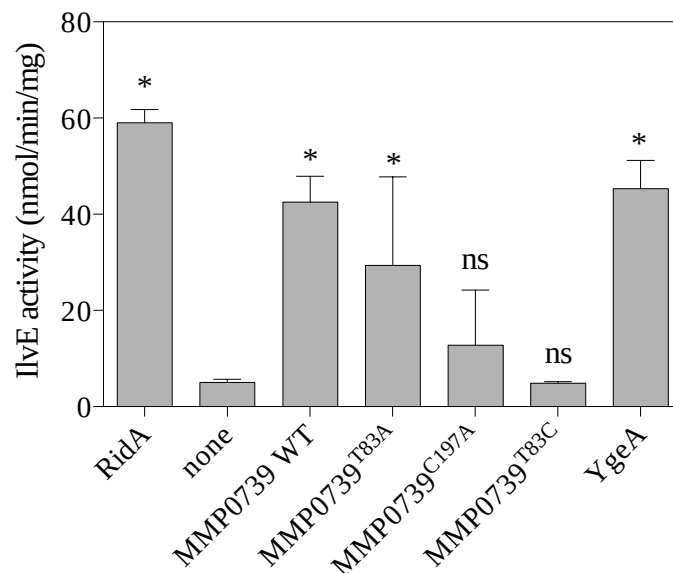


Figure 4.8 MMP0739 improves IlvE activity in a *ridA* strain

Strains lacking *ridA* (DM12920) were transformed with pBAD24 constructs encoding *Se* RidA, MMP0739 wild type or T83C, T83A, C197A variants, *Se* YgeA or an empty vector control. Cultures were grown in minimal medium with glycerol (30 mM) as the sole carbon source with arabinose (0.2%) until all cultures reached full density. IlvE activity was assayed as described in the text. Error bars indicate the standard deviation of crude extract from three biological replicates and data are representative from at least two independent experiments. The specific activity of IlvE of each strain expressing a recombinant protein was compared to the reduced activity in *ridA* mutant strain (none) using a one-way ANOVA and significant values are indicated with * ($p < 0.05$).

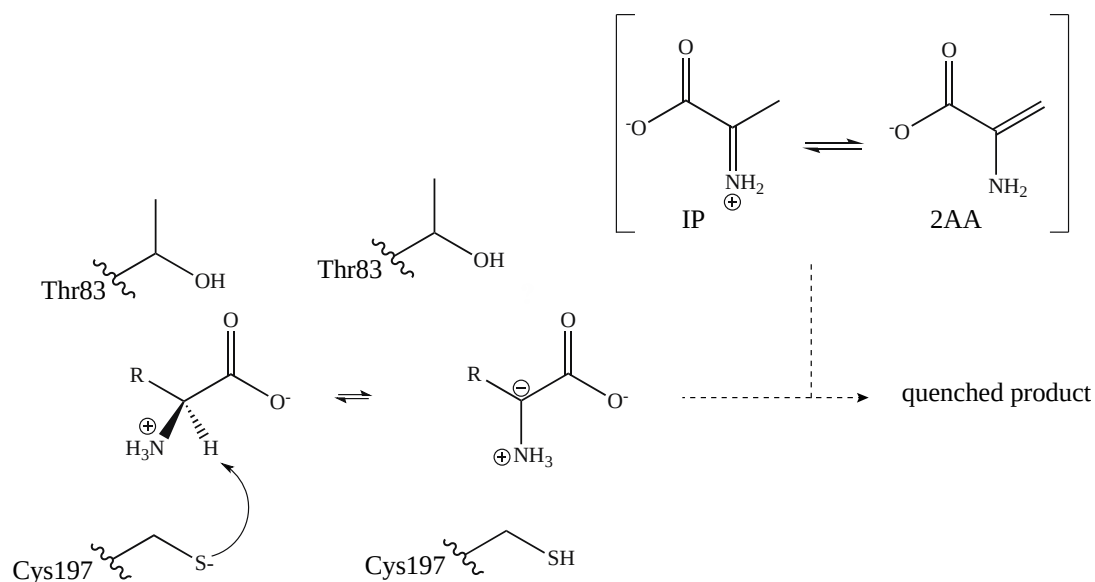


Figure 4.9 Model for indirect 2-aminoacrylate quenching. A model for 2AA quenching with a metabolite-mediated mechanism is depicted. In this scenario, a PLP-independent racemase with a threonine/cysteine catalytic pair (i.e., MMP0739 or YgeA) catalyzes the formation of an intermediate with a carbanion from an unknown amino acid substrate. Lack of the canonical racemase proton donor (cysteine) is likely to slow conversion of the intermediate to the substrate antipode. We suggest 2AA, or the iminopropionate (IP) tautomer, reacts with the carbanion in either an enzyme-bound or free state, which results in formation of an adduct that prevents 2AA from damaging cellular targets.

CHAPTER 5

CHARACTERIZING THE MUTANT PHENOTYPE OF A *PSEUDOMONAS AERUGINOSA* STRAIN LACKING THE RIDA HOMOLOG PA5339¹

¹ Hodge-Hanson K. M. and D. M. Downs. *To be submitted to Journal of bacteriology*

5.1 ABSTRACT

The Rid protein superfamily (YjgF/YER057c/UK114) is found in all domains of life. The archetypal protein, RidA from *Salmonella enterica*, is a deaminase that quenches the reactive metabolite 2-aminoacrylate (2AA). 2AA deaminase activity is conserved in RidA proteins from humans, plants, yeast, archaea and bacteria. Mutant strains of multiple organisms that lack a functional RidA exhibit growth defects, suggesting 2AA metabolic stress is similarly conserved. In contrast to *S. enterica* with one RidA, *Pseudomonas aeruginosa* encodes a total of four RidA homologs, all of which are predicted to deaminate 2AA. Mutant strains of *P. aeruginosa* PAO1 lacking each of the four RidA proteins were screened, and the mutant phenotype that arose in the absence of PA5339 was dissected via nutritional analysis. PA5339 demonstrated 2AA deaminase activity *in vitro* and restored metabolic balance to a *S. enterica ridA* strain. The growth defects that arise when RidA is inactivated in multiple organisms manifest as a result of 2AA stress. The experiments described here probed whether that the mutant phenotype of a Δ PA5339 strain similarly stemmed from 2AA accumulation.

5.2 INTRODUCTION

Members of the RidA subfamily of the Rid (YjgF/YER057c/UK114) protein superfamily are found in all domains of life, and prokaryotic genomes often encode multiple proteins from various subfamilies (RidA and Rid1-7). The archetypal protein, RidA from *Salmonella enterica*, deaminates the reactive metabolite 2-aminoacrylate, which arises as a catalytic intermediate from a number of pyridoxal 5'-phosphate (PLP)-dependent reactions (1-3) (Figure 5.1). To date, all RidA proteins tested, including human, plant, and archaeal homologs, have demonstrated 2AA deaminase activity, suggesting the role in quenching metabolic stress is conserved (4, 5). Although the pleiotropic phenotypes of a *S. enterica* strain lacking *ridA* stem from the

accumulation of 2AA, RidA proteins deaminate a wide range of substrates (6, 7). *S. enterica* encodes a single RidA protein and two additional members of the Rid superfamily that do not quench 2AA (7). In contrast, *Pseudomonas* strains encode four RidA homologs that belong to the RidA subfamily as well as multiple Rid1 and Rid2 subfamily proteins (Figure 5.2). Based on the activity of diverse RidA subfamily proteins tested previously (4), the expectation was that each of the *Pseudomonas* RidA proteins would catalyze 2AA deamination, and had potential to restore metabolic balance to an *S. enterica* *ridA* strain.

Growth defects have been reported in other organisms that lack the relevant RidA homolog, including yeast (8, 9) and plants (5, 10), and are restored by the addition of isoleucine. In *Salmonella enterica*, the 2AA burden leads to metabolic imbalance in a *ridA* mutant strain that can be restored through supplementation with isoleucine or glycine (11, 12). Previous work showed that the amino acids were not nutritionally required and instead circumvented 2AA cellular damage through a distinct mechanism (3, 13, 14). Isoleucine allosterically inhibited IlvA, the major 2AA generator in *S. enterica*, while glycine bypassed the damaged GlyA enzyme by allowing regeneration of 1-carbon units via the glycine cleavage complex. Therefore, all of the reported mutant phenotypes that arise in the absence of a functional RidA protein are expected to stem from 2AA accumulation.

Members of the Rid superfamily have been the subjects of structure-function analyses, including PA3499 from *P. aeruginosa* (PDB 2IG8), for which a structure has been solved but a functional role in *P. aeruginosa* remains to be elucidated. As the number of sequenced genomes increases at a rate that cannot be matched by experimental validation, misannotations are propagated at a worrisome rate (6, 15, 16). The RidA proteins provide an excellent example of an enzyme class in the early stages of annotation following the elucidation of the 2AA stress

paradigm in *S. enterica* (4, 12, 17, 18). This study describes the framework components of the 2AA stress paradigm that are present in *P. aeruginosa*, and dissects the mutant phenotype of a *P. aeruginosa* strain lacking the RidA subfamily protein PA5339. Additionally, these data challenge the prediction that the collective group of RidA proteins in *P. aeruginosa* share the same functional role.

5.3 MATERIALS AND METHODS

Bacterial strains, plasmids and media.

The strains and plasmids used in this study are listed in Table 5.1. *Pseudomonas aeruginosa* PAO1 strains were obtained from the transposon mutant library collection (19), including the wild type strain MPAO1. Transposon location was verified using a transposon-specific primer and a primer annealing to a flanking region of the chromosome. Table 5.2 lists the primers used in this study. *Salmonella enterica* strains were derivatives of *S. enterica* serovar Typhimurium LT2 and *Escherichia coli* BL21AI was used for protein overexpression.

Lysogeny broth (LB) was used as a rich medium for *P. aeruginosa* and *E. coli*. *S. enterica* was cultivated in Difco Nutrient Broth (8 g/liter) with NaCl (5 g/liter). All bacterial strains were grown at 37°C. MOPS salts (20) or NCE with MgSO₄ (21) and trace minerals (22) were used as a minimal medium base, as indicated. *P. aeruginosa* strains were grown with either glucose or succinate as the sole carbon source, added at a final concentration of 20 mM. Glycerol (30 mM) was used as the carbon source for *S. enterica* strains. Gentamicin was added to 100 µg/mL for *P. aeruginosa* and 10 µg/mL for *S. enterica*. Ampicillin was added to 150 µg/mL for *S. enterica* and *E. coli*. Supplements were added at the following concentrations: isoleucine, 0.3 mM; threonine, 0.3 mM; glycine, 1 mM; serine, 5 mM; and cysteine, 250 µM. L-arabinose was added to growth studies to induce relevant plasmids at various concentrations (0.02-0.2%).

Chemicals were purchased from MilliporeSigma (Sigma-Aldrich, St. Louis, MO) and yeast media reagents were purchased from Sunrise Scientific (San Diego, CA).

Growth analysis

Growth on solid medium was evaluated by patching strains to rich medium (LB), incubating plates for 3-4 hours at 37°C, and printing to agar plates containing minimal medium using sterile velvets and a printing block. Printed plates were incubated at 37°C overnight before evaluating growth or imaging. Top agar overlays on agar plates containing molten agar (0.77 g/liter) and an aliquot of overnight culture (100 µL) that had been pelleted and resuspended in sterile saline solution.

Growth in liquid culture was assessed using a BioTek Elx808 microtiter plate reader via optical density at 650 nm. Overnight cultures of *P. aeruginosa* were grown in rich medium (2 mL), pelleted and resuspended in an equal volume of sterile NCE (1x) salts. An aliquot (2 µL) of the cell resuspension was used to inoculate the growth medium (0.2 mL). Overnight cultures of *S. enterica* were grown in rich medium (2 mL) and were directly used to inoculate growth medium (2% inoculum). Cultures were grown in biological triplicate at 37°C with slow shaking speed and growth curves were plotted using GraphPad Prism (version 6.0g).

Molecular biology

An *in vivo* recombineering protocol was used to clone into the pMQ72 vector based on the Gibson cloning method and an approach described by Shanks, *et al.* (23-27). Primers were designed to amplify genes of interest with ~40 bp homology to the pMQ72 plasmid. Purified PCR products (~20 µL) were mixed with single-stranded salmon sperm carrier DNA (40 µg in TE buffer) and pMQ72 plasmid DNA (5 µL) that had been linearized via *Sma*I digestion. The DNA mixture was transformed into an aliquot of yeast cells (0.5 mL) that had grown to full

density in YPD medium (yeast extract, 10 g/liter; peptone, 20 g/liter ; glucose, 2%), was pelleted via centrifugation and resuspended in an equal volume of Lazy Bones Solution (40% polyethylene glycol, molecular weight 3350; 0.1 M lithium acetate; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA). The yeast transformation was incubated at room temperature for 24 hours followed by a heat shock (10 minutes) at 42°C. Cells were pelleted, the supernatant was discarded and the cell pellet was resuspended in sterile TE buffer (100 µL) and spread onto selective medium. Prototrophic yeast strains were selected on SD medium lacking uracil (Yeast Nitrogen Base plus vitamins, 1.7g/liter; ((NH₄)₂SO₄, 5 g/liter; amino acid mixture lacking uracil, 2 g/liter; agar, 20 g/liter, glucose, 2%). Uracil prototrophs were pooled and plasmids were isolated using a PureYield Plasmid Miniprep System (Promega) and glass beads (425-600 µm) to lyse yeast cells. The isolated plasmid pool was electroporated into *E. coli* and transformants were selected on LB containing gentamicin. Plasmid inserts were screened via colony PCR with primers PR923 and PR924. Candidate constructs were confirmed via sequence analysis performed by Eton Biosciences (San Diego, CA). Plasmid derivatives of pBAD24 were created using a BspQI restriction cloning method as previously described by Galloway, *et al.* (28) with a modified vector that contained the BspQI site (pCV1) (29). *P. aeruginosa* competent cells were prepared by washing overnight cultures (1 mL) in sucrose (0.3 M) three times and resuspending in a final volume of sucrose solution (80 µL). Competent cells were transformed with plasmids using electroporation and transformants were recovered in LB (1 mL) and plated to selective medium at 37°C.

Protein purification

PA5339 was purified from *E. coli* strain BL21AI harboring pDM1563 (pET28-PA5339) based on the RidA purification scheme described previously (30). Briefly, overnight cultures in

LB (10 mL) were used to inoculate 2 flasks containing a total of 3 liters super broth supplemented with ampicillin. Cultures were grown at 37°C with shaking (200 rpm) until the optical density at 650 nm reached 0.5. Protein overexpression was induced with the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG, 0.1 mM) and arabinose (0.2% w/v), and culture temperature was shifted to 30°C overnight. Cells were harvested via centrifugation at 6,000 x g for 15 minutes, and the cell pellets were kept at -80°C until use. A volume of bind buffer (50 mM potassium phosphate, pH 7.5; 100 mM NaCl; 5 mM imidazole and 10% glycerol) was added to the cell pellets (2 mL per gram wet cell weight) along with lysozyme (1 mg/mL), DNase (20 Units/mL) and phenylmethanesulfonyl fluoride PMSF (1 mM). Cells were lysed with a cell disruptor using the 'One Shot Head' (Constant Systems, LTD, Northants, United Kingdom) set to 20 kpsi, and debris was pelleted via centrifugation at 40,000 x g for 40 minutes. The lysate was clarified with a syringe filter (0.45 micron) prior to being loaded onto a 5 mL HisTrap HP (Amersham Biosciences) column on an ÄKTA FPLC. Protein was eluted with a 0-100% gradient of elution buffer (50 mM potassium phosphate, pH 7.5; 100 mM NaCl; 0.5 M imidazole and 10% glycerol) per the manufacturer's instruction (GE Healthcare). Samples (2 μ L) were collected from the elution fractions, analyzed via SDS-PAGE, and desired fractions were identified based on the presence of a ~ 13 kDa band. The PA5339 fractions were pooled and concentrated using a centrifugal protein concentrator unit with a 3,000 MWCO (Millipore-Sigma). The protein was transferred into storage buffer (50 mM potassium phosphate, pH 7.5; 10% glycerol) with a PD-10 desalting column (GE Healthcare). PA5339 concentration was evaluated using the BCA protein assay kit (Pierce), and dilutions (0.125-2 μ g) were loaded onto a 12.5% SDS-PAGE gel for protein purity analysis. PA5339 was enriched to greater than 99% purity and the preparation was drop-frozen in liquid nitrogen and stored at -80°C.

Cysteine desulfhydrase (CdsH) assays

2AA deaminase activity was assayed using a coupled assay with purified cysteine desulfhydrase-dependent pyruvate formation and NADH oxidation by lactate dehydrogenase as previously described (2). Reaction mixtures (100 μ L) contained Tris-HCl (100 mM, pH 8), NADH (250 μ M), pyridoxal 5'-phosphate (30 μ M), and pyruvate kinase/lactate dehydrogenase (5 Units). Purified cysteine desulfhydrase (CdsH) and the indicated Rid proteins were added at monomeric concentrations of 0.27 μ M or 0.19 μ M respectively. The reaction was initiated with addition of freshly prepared L-cysteine at final concentrations between 0.2 and 2 mM. Absorbance at 340 nm was monitored for two minutes and the initial rate of pyruvate formation was calculated from the rate of NADH oxidation in the first 30 seconds along with the molar extinction coefficient for NADH oxidation (6,200 M⁻¹ cm⁻¹).

5.4 RESULTS

A single mutation in PA5339 elicits a growth defect in *Pseudomonas aeruginosa*

Mutant strains of *Pseudomonas aeruginosa* PAO1 that lacked each of the Rid proteins listed in Table 1 were screened for growth defects on a variety of minimal media. Growth was tested on medium containing NCE salts with glucose as the sole carbon source or MOPS with either glucose or succinate. The media were chosen based on reported minimal media used for *P. aeruginosa* cultivation (31, 32). On each of the media tested, strains lacking PA0814, PA1568, PA3123, PA3449, PA5303, or PA5392 were prototrophic, while a single mutation in PA5339 prevented growth on minimal medium when the strains were patched to rich (LB) medium and printed to minimal medium (Table 5.3). This was surprising, given the subtlety of the pleiotropic phenotypes associated with an *S. enterica* *ridA* mutant (11, 13, 14, 17). Additionally, the Δ PA5339 mutant phenotype suggested RidA homologs PA3123, PA3499 and PA5392 did not

substitute for PA5339 activity under the conditions tested. Non-RidA subfamily proteins PA0814 (Rid1) and PA5083 (Rid2) demonstrated 2AA deaminase activity *in vitro* and *in vivo* (7), and these results suggested the Rid1 or Rid2 subfamily proteins were not compensatory under the conditions tested. The growth defect of a *P. aeruginosa* Δ PA5339 mutant strain was dissected using nutritional analysis. Based on the observation that growth defects manifest in multiple organisms in the absence of a RidA subfamily protein as a result of 2AA stress (5, 8-11), the Δ PA5339 mutant phenotype was explored.

Nutritional studies identify amino acids that restore growth of a Δ PA5339 mutant strain

In order to identify which area(s) of metabolism were affected in the absence of PA5339, a general nutritional screen was performed with vitamins and amino acids. Strains lacking PA5339 (PW9994 and PW9995) were embedded in a top agar overlay on plates containing NCE with glucose as the sole carbon source. Overnight cultures grown in rich medium were not washed prior to addition to the top agar, and faint growth of the Δ PA5339 mutant strain was visible after overnight incubation. By comparison, a similar top agar containing wild type *P. aeruginosa* produced a dense lawn. Pools and individual stocks of compounds, including all twenty proteinogenic amino acids, were spotted and evaluated for stimulatory or inhibitory effects on growth of Δ PA5339 mutant strains (PW9994 and PW9995). A pool containing isoleucine, thiamine, valine, proline, arginine, glycine and alanine stimulated growth. Individual components of the pool were evaluated, and either isoleucine or thiamine alone improved growth on plates. In addition, threonine was stimulatory, and serine inhibited growth of both Δ PA5339 mutant strains (PW9994 and PW9995) but did not inhibit growth of the wild type.

Individual compounds that produced an effect on plates were evaluated with Δ PA5339 strains growing in liquid NCE medium containing glucose as the sole carbon source. The

Δ PA5339 mutant strains (PW9994 and PW9995) displayed a growth defect relative to wild type in liquid medium but reached similar final optical densities after a lag period. However, the apparent difference between growth of the mutant and wild type was not as significant in liquid compared to the patch plate phenotypes. Figure 5.3 shows a patch plate containing of the tested *P. aeruginosa* strains without or with isoleucine added. The growth difference between solid and liquid media suggested that aeration might influence growth of the Δ PA5339 strain, but this possibility was not explored further. Both Δ PA5339 mutant strains (PW9994 and PW9995) had similar growth profiles in liquid medium (data not shown) and PW9994 was selected for further characterization. Supplementation with isoleucine or threonine improved growth to near wild type levels, and glycine partially restored growth (Figure 5.4A). Each of these amino acids restores metabolic balance in a *S. enterica* *ridA* mutant strain by either inhibiting the production of 2AA or circumventing 2AA damage (3, 11, 12, 33). The partial growth restoration by glycine mirrored the *S. enterica* paradigm, in which 2AA leads to a one-carbon limitation as a result of inactivated serine hydroxymethyltransferase (3, 12). Serine hydroxymethyltransferase is highly conserved in free-living organisms (34), and *P. aeruginosa* encodes multiple serine hydroxymethyltransferase isozymes. If glycine provides an analogous benefit in *P. aeruginosa* to its defined role in *S. enterica*, we expect that serine hydroxymethyltransferase activity will be diminished in a Δ PA5339 mutant strain.

Potential 2AA sources are present in *P. aeruginosa*

In *S. enterica*, 2AA is produced *in vivo* by fold-type II pyridoxal 5'-phosphate-dependent enzymes, including serine/threonine dehydratase IlvA (EC 4.3.1.19) with substrate serine (1, 4, 18). The inhibitory effect of serine on growth of Δ PA5339 mutant strains described above was consistent with 2AA generation by a serine/threonine dehydratase. It is plausible that isoleucine

or threonine restore growth via allosteric (isoleucine) or competitive (threonine) inhibition of one or both of the *P. aeruginosa* IlvA homologs encoded by *ilvA1* and *ilvA2*. PLP-dependent serine/threonine dehydratase activity was identified in *P. aeruginosa* PAC1, and following purification, the active fraction was highly sensitive to inhibition to isoleucine (35). Further, both *P. aeruginosa* PAO1 isozymes retain the conserved allosteric regulatory region of *S. enterica* IlvA. The *P. aeruginosa* genome additionally encodes two putative threonine/serine dehydratases that lack this regulatory region, as does the *S. enterica* catabolic isozyme TdcB, which is expressed anaerobically and regulated differently than IlvA (36, 37). An alternative explanation for the Δ PA5339 mutant growth defect is that an isoleucine limitation exists, and that supplementation with threonine increases flux to isoleucine biosynthesis. Either of these two scenarios is a plausible explanation for the stimulatory effect of isoleucine or threonine on growth of a Δ PA5339 mutant strain.

2AA precursor amino acids inhibit growth of Δ PA5339 mutant

In addition to the serine/threonine dehydratases, the *P. aeruginosa* genome encodes multiple putative cysteine desulfhydrase isozymes (EC 2.5.1.47) (Table 5.4). *S. enterica* *ridA* strains growing in liquid minimal medium exhibit a slight growth defect that is exacerbated by supplementation with 2AA precursors (serine and cysteine) (3, 12). The potential for *P. aeruginosa* serine/threonine dehydrogenase or putative cysteine desulfhydrase enzymes to exacerbate the growth defect in liquid was probed by addition of exogenous 2AA precursors (serine and cysteine) to minimal medium supplemented with isoleucine. Although exogenous cysteine poses a general risk to bacterial growth, this is minimized under dilute concentrations. At 250 μ M, cysteine does not affect growth of wild type *S. enterica*, but does exacerbate 2AA accumulation in a *ridA* mutant strain (2). In order to rule out the effects of general toxicity, serine

and cysteine were added at concentrations that had no effect on growth of wild type *P. aeruginosa* (data not shown). A Δ PA5339 mutant strain (PW9994) was grown in minimal medium containing isoleucine and supplemented with serine or cysteine (Figure 5.4B). In combination with isoleucine, which restored growth alone, serine and cysteine inhibited growth of the Δ PA5339 mutant, which was consistent with the generation of 2AA by serine/threonine dehydratase and cysteine desulfhydrase activity, respectively. Importantly, these results distinguish *P. aeruginosa* Δ PA5339 mutant strains from *S. enterica* *ridA* mutants and suggest that *P. aeruginosa* differs from the *S. enterica* 2AA stress paradigm. Isoleucine supplementation of a *S. enterica* *ridA* strain abolishes the effect of serine due to inactivation of *IlvA*. If the relevant role of isoleucine in restoring growth to a Δ PA5339 mutant strain is via allosteric regulation of *IlvA* isozymes, then it is possible that an alternative enzyme might generate 2AA under these conditions. It is possible that additional 2AA is produced by either of the *TdcB* homologs (PA0851 or PA2683) or a PLP-dependent enzyme that proceeds through a 2AA catalytic intermediate. This scenario might be addressed by additional characterization of the *IlvA* and *TdcB* isozymes. Further, if isoleucine serves an exclusively allosteric role, the expectation is that isoleucine will not restore growth in a Δ PA5339 mutant harboring a feedback-resistant *ilvA* allele (14).

Complementation study indicates PA5339 mutation is causative

To confirm the mutation in PA5339 was causative, pDM1533 (pMQ72-PA5339) was constructed and tested for the ability to restore growth of the Δ PA5339 mutant strains. Δ PA5339 mutant strains (PW9994 and PW9995) were transformed with pDM1533 or pMQ72 lacking an insert and growth of the resulting strains was assessed in minimal (NCE) medium containing glucose as the sole carbon source. pDM1533 alone restored growth to both PW9994 (Figure

5.5A) and PW9995 (data not shown) without induction. The pMQ72 constructs are inducible by arabinose (23), and compared to medium that lacked arabinose, there was no difference in growth when varying concentrations of arabinose (0.02-0.2%) were added to the growth medium. The data showed that the mutation in PA5339 was causative.

A subset of RidA proteins improve growth of PW9994

The growth defect elicited by a single mutation in PA5339 prompted evaluation of the additional PAO1 RidA subfamily proteins (PA3123, PA3449 and PA5392), as well as *S. enterica* RidA on a high-copy inducible vector in a Δ PA5339 mutant strain. It seemed plausible that differences in transcription might prevent compensatory activity. Furthermore, if introduction of a functional 2AA deaminase such as *S. enterica* RidA improved growth, it would indicate the activity was absent in a Δ PA5339 mutant strain. A strain lacking PA5339 (PW9994) was transformed with pMQ72 constructs harboring PA5392 (pDM1532), PA5339 (pDM1533), PA3123 (pDM1566), *S. enterica* RidA (pDM1568) or no insert (pMQ72). Growth of the resulting strains was monitored in minimal (NCE) medium containing glucose as the sole carbon source. Without induction by arabinose, expression of *S. enterica* RidA or PA5339 fully restored growth relative to the empty vector control (Figure 5.5A). PA5392 did not substitute for the loss of PA5339, and seemed to confer a slight growth defect in each of the media tested (Figure 5.5). Growth of the Δ PA5339 strains was similar without or with arabinose (data not shown) and discrepancies in protein abundance in these strains were not investigated further. The heterologous complementation by a demonstrated enamine deaminase, *S. enterica* RidA, suggested this was the relevant activity abolished by the PA5339 mutation in PW9994. The addition of isoleucine universally improved growth (Figure 5.5B).

The effect of isoleucine and serine in combination was probed using the Δ PA5339 mutant strains harboring pMQ72 constructs. In comparison to growth in minimal (NCE) medium with glucose as the sole carbon source (Figure 5.5A), growth of the empty vector control strain was inhibited by addition of serine to the medium. pDM1533 (PA5339) and pDM1568 (*S. enterica* RidA) complemented the growth defect, and pDM1566 (PA3123) conferred a slight growth benefit (Figure 5.5C). pDM1532 (PA5392) did not improve growth. Isoleucine supplementation improved growth of each of these strains in medium containing serine (Figure 5.5D). These results were consistent with a scenario in which generation of 2AA from serine by a serine/threonine dehydratase prevents growth of Δ PA5339 mutant strains. The partial restoration of growth with isoleucine supplementation suggests that an additional generator may be involved.

Select *P. aeruginosa* RidA proteins deaminate 2AA *in vivo*

The pleiotropic phenotypes of an *S. enterica ridA* strain stem from 2AA accumulation (4, 12, 17, 18) and can be overcome by providing a functional 2AA deaminase *in trans* in the standard *in vivo* assay for RidA activity (5, 7). Each of the four RidA proteins from *P. aeruginosa* were evaluated for the ability to restore metabolic balance to an *S. enterica ridA* mutant strain. A *S. enterica ridA* strain (DM12920) was transformed with pBAD24 vectors encoding each of the four RidA homologs. The resulting strains, along with DM14846 (pBAD24-*ridA*) and DM14847 (pBAD24), were assessed for the ability to grow in minimal NCE medium containing glycerol and serine. The growth curves are presented in Figure 5.6. pDM1559 (pBAD24-PA5339), pDM1559 and pDM1561 (pBAD24-PA3123) restored metabolic balance in a *ridA* strain with 0.02% arabinose induction. pDM1560 (pBAD24-PA5392), and pDM1562 (pBAD24-PA3499) were unable to restore balance to an *S. enterica ridA* strain at any

level of induction, and growth restoration by pDM1559 (pBAD24-PA5339) did not require arabinose addition (data not shown). The *S. enterica* complementation data indicated that PA5339 and PA3123 have 2AA deaminase activity. Although 2AA deaminases from each domain of life substituted for RidA *in vivo*, a survey of *Pseudomonas* Rid1, Rid2 and Rid3 proteins (from *P. aeruginosa*, *P. fluorescens*, or *P. syringae*) found that all five of the tested proteins were active deaminases *in vitro*, yet only three were active *in vivo* (7). As with the other *Pseudomonas* Rid proteins, the inability of PA3499 or PA5392 to restore metabolic balance to a *S. enterica ridA* mutant strain might indicate protein stability or folding issues, which could account for the lack of complementation. It is also possible that PA3499 and PA5392 serve alternative roles in *P. aeruginosa* and may not substantially deaminate 2AA *in vivo*.

P. aeruginosa* PA5339 deaminates 2AA *in vitro

Purified PA5339 was assayed using a RidA activity assay that uses a purified cysteine desulfhydrase (CdsH) to generate 2AA (2). Due to the short half-life of 2AA, the standard RidA assay relies on activity of a purified PLP-dependent enzyme in order to generate 2AA *in situ*. CdsH converts cysteine to 2AA, which is then deaminated by water to form pyruvate. The rate of pyruvate formation can be coupled to lactate dehydrogenase activity and monitored at an absorbance of 340 nm. Addition of RidA leads to an increased rate of pyruvate formation, indicating 2AA deaminase activity. Addition of PA5339 to the reaction mixture led to an increased rate of pyruvate formation at multiple concentrations of cysteine (0.2-2 mM), which was similar to the activity of *S. enterica* RidA (Figure 5.7). These data, combined with complementation of a *S. enterica ridA* strain, showed that PA5339 is a 2AA deaminase.

5.5 CONCLUSIONS

The *P. aeruginosa* genome encodes nine Rid superfamily proteins, four of which belong to the RidA subfamily and are therefore predicted to be 2AA deaminases. PA5339 demonstrated 2AA deaminase activity *in vitro* and *in vivo* (*S. enterica*). A single mutation in PA5339 elicited a growth defect on multiple types of minimal medium, and this mutant phenotype was explored in the context of 2AA stress. Complementation of the PA5339 growth defect by two functional 2AA deaminases (PA5339 and *S. enterica* RidA), combined with the exacerbated growth defect when a 2AA-precursor (serine or cysteine) was added to the growth medium, were consistent with a scenario where the mutant phenotype arises as a result of 2AA accumulation. Initial studies in *P. aeruginosa* suggest differences compared to the 2AA paradigm established in *S. enterica*.

The idea that 2AA stress could differ between organisms was realized in a comparison of the *E. coli* and *S. enterica* metabolic networks, where the consequences of a *ridA* mutation are different despite conservation of relevant proteins with similar kinetic properties (38). Isoleucine biosynthesis is the major 2AA source in these organisms, and in contrast to the enteric operon structure, the isoleucine biosynthetic genes in *P. aeruginosa* are not clustered (39). *P. aeruginosa* encodes multiple isozymes of relevant 2AA generators and targets, for which the regulatory mechanisms are not fully understood. A hallmark of 2AA damage in *S. enterica* *ridA* strains is the decreased activity of isoleucine transaminase, which did not seem to manifest in *P. aeruginosa* Δ PA5339 mutant strains (data not shown). Supplementation with glycine suggested a defect in serinehydroxymethyltransferase activity, but the partial restoration was consistent with inactivation of additional 2AA targets.

Pseudomonas genomes encode multiple Rid proteins and elucidation of each functional role will likely uncover new metabolic connections and support elucidation of the putative 2AA generator and target enzymes. Most *P. aeruginosa* strains lacking a Rid protein were prototrophic on multiple types of minimal media. A Δ PA5083 mutant strain was not included in this initial screen as it was not available in the collection. PA5083 has been validated as a 2AA deaminase *in vitro* and mediates 2AA stress in a *S. enterica* *ridA* mutant strain. PA5083 is encoded near several putative D-glutamate utilization genes, and its *in vitro* activity suggested a role in facilitating imine to ketoacid conversion in D-amino acid metabolism (7). Continued phenotypic screening of strains lacking each of the Rid proteins, including a Δ PA5083 mutant strain, will contribute to the understanding the role(s) of Rid proteins from multiple subfamilies.

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5.7 REFERENCES

1. Lambrecht JA, Flynn JM, Downs DM. Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions. *J Biol Chem.* 2012;287(5):3454-61.
2. Ernst DC, Lambrecht JA, Schomer RA, Downs DM. Endogenous synthesis of 2-aminoacrylate contributes to cysteine sensitivity in *Salmonella enterica*. *Journal of bacteriology.* 2014;196(18):3335-42.
3. Ernst DC, Anderson ME, Downs DM. L-2,3-diaminopropionate generates diverse metabolic stresses in *Salmonella enterica*. *Mol Microbiol.* 2016;101(2):210-23.
4. Lambrecht JA, Schmitz GE, Downs DM. RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life. *MBio.* 2013;4(1):e00033-13.
5. Niehaus TD, Nguyen TN, Gidda SK, ElBadawi-Sidhu M, Lambrecht JA, McCarty DR, et al. Arabidopsis and maize RidA proteins preempt reactive enamine/imine damage to branched-chain amino acid biosynthesis in plastids. *Plant Cell.* 2014;26(7):3010-22.

6. Niehaus TD, Gerdes S, Hodge-Hanson K, Zhukov A, Cooper AJ, ElBadawi-Sidhu M, et al. Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC Genomics*. 2015;16:382.
7. Hodge-Hanson KM, Downs DM. 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*. 2017;12(9):e0185544.
8. Kim JM, Yoshikawa H, Shirahige K. A member of the YER057c/yjgF/Uk114 family links isoleucine biosynthesis and intact mitochondria maintenance in *Saccharomyces cerevisiae*. *Genes to cells : devoted to molecular & cellular mechanisms*. 2001;6(6):507-17.
9. Oxelmark E, Marchini A, Malanchi I, Magherini F, Jaquet L, Hajibagheri MA, et al. Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome. *Molecular and cellular biology*. 2000;20(20):7784-97.
10. Leitner-Dagan Y, Ovadis M, Zuker A, Shklarman E, Ohad I, Tzfira T, et al. CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. *Planta*. 2006;225(1):89-102.
11. Enos-Berlage JL, Langendorf MJ, Downs DM. Complex metabolic phenotypes caused by a mutation in yjgF, encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of bacteriology*. 1998;180(24):6519-28.
12. Flynn JM, Christopherson MR, Downs DM. Decreased coenzyme A levels in ridA mutant strains of *Salmonella enterica* result from inactivated serine hydroxymethyltransferase. *Mol Microbiol*. 2013;89(4):751-9.
13. Schmitz G, Downs DM. 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. *Journal of bacteriology*. 2004;186(3):803-10.
14. Christopherson MR, Schmitz GE, Downs DM. YjgF is required for isoleucine biosynthesis when *Salmonella enterica* is grown on pyruvate medium. *J Bacteriol*. 2008;190(8):3057-62.
15. Schnoes AM, Brown SD, Dodevski I, Babbitt PC. Annotation error in public databases: misannotation of molecular function in enzyme superfamilies. *PLoS Comput Biol*. 2009;5(12):e1000605.
16. Babbitt PC. Definitions of enzyme function for the structural genomics era. *Current opinion in chemical biology*. 2003;7(2):230-7.
17. Flynn JM, Downs DM. In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5'-phosphate cofactor. *J Bacteriol*. 2013;195(16):3603-9.

18. Downs DM, Ernst DC. From microbiology to cancer biology: the Rid protein family prevents cellular damage caused by endogenously generated reactive nitrogen species. *Molecular microbiology*. 2015;96(2):211-9.
19. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, et al. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*. 2003;100(24):14339-44.
20. Neidhardt FC, Bloch PL, Smith DF. Culture medium for enterobacteria. *Journal of bacteriology*. 1974;119(3):736-47.
21. Vogel HJ, Bonner DM. Acetylornithinase of *Escherichia coli*: partial purification and some properties. *The Journal of biological chemistry*. 1956;218(1):97-106.
22. Balch WE, Wolfe RS. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Applied and environmental microbiology*. 1976;32(6):781-91.
23. Shanks RM, Caiazza NC, Hinsa SM, Toutain CM, O'Toole GA. *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria. *Appl Environ Microbiol*. 2006;72(7):5027-36.
24. Gibson DG. Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol*. 2011;498:349-61.
25. Burke D, Dawson D, Stearns T, Cold Spring Harbor Laboratory. *Methods in yeast genetics : a Cold Spring Harbor Laboratory course manual*. 2000 ed. Plainview, N.Y.: Cold Spring Harbor Laboratory Press; 2000. xvii, 205 p. p.
26. Elble R. A simple and efficient procedure for transformation of yeasts. *Biotechniques*. 1992;13(1):18-20.
27. Oldenburg KR, Vo KT, Michaelis S, Paddon C. Recombination-mediated PCR-directed plasmid construction in vivo in yeast. *Nucleic Acids Res*. 1997;25(2):451-2.
28. Galloway NR, Toutkoushian H, Nune M, Bose N, Momany C. Rapid Cloning For Protein Crystallography Using Type IIS Restriction Enzymes. *Crystal Growth & Design*. 2013;13(7):2833-9.
29. VanDrisse CM, Escalante-Semerena JC. New high-cloning-efficiency vectors for complementation studies and recombinant protein overproduction in *Escherichia coli* and *Salmonella enterica*. *Plasmid*. 2016;86:1-6.
30. Lambrecht JA, Browne BA, Downs DM. Members of the YjgF/YER057c/UK114 family of proteins inhibit phosphoribosylamine synthesis in vitro. *J Biol Chem*. 2010;285(45):34401-7.

31. Dietrich LE, Price-Whelan A, Petersen A, Whiteley M, Newman DK. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol Microbiol*. 2006;61(5):1308-21.
32. Silo-Suh L, Suh SJ, Phibbs PV, Ohman DE. Adaptations of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment can include deregulation of *zwf*, encoding glucose-6-phosphate dehydrogenase. *J Bacteriol*. 2005;187(22):7561-8.
33. Christopherson MR, Lambrecht JA, Downs D, Downs DM. Suppressor analyses identify threonine as a modulator of *ridA* mutant phenotypes in *Salmonella enterica*. *PloS one*. 2012;7(8):e43082.
34. Ernst DC, Downs DM, Metcalf WW. 2-Aminoacrylate Stress Induces a Context-Dependent Glycine Requirement in *ridA* Strains of *Salmonella enterica*. *Journal of Bacteriology*. 2016;198(3):536-43.
35. Lam VMS, Yeung YG. Properties of threonine deaminase from *Pseudomonas aeruginosa*. *FEMS Microbiol Letters*. 1978;3:219-21.
36. Umbarger HE, Brown B. Threonine deamination in *Escherichia coli*. II. Evidence for two L-threonine deaminases. *J Bacteriol*. 1957;73(1):105-12.
37. Luginbuhl GH, Hofler JG, Decedue CJ, Burns RO. Biodegradative L-threonine deaminase of *Salmonella typhimurium*. *J Bacteriol*. 1974;120(1):559-61.
38. Borchert AJ, Downs DM. The Response to 2-Aminoacrylate Differs in *Escherichia coli* and *Salmonella enterica*, despite Shared Metabolic Components. *J Bacteriol*. 2017;199(14).
39. Vitreschak AG, Lyubetskaya EV, Shirshin MA, Gelfand MS, Lyubetsky VA. Attenuation regulation of amino acid biosynthetic operons in proteobacteria: comparative genomics analysis. *FEMS Microbiol Lett*. 2004;234(2):357-70.
40. Kim KS, Pelton JG, Inwood WB, Andersen U, Kustu S, Wemmer DE. The Rut pathway for pyrimidine degradation: novel chemistry and toxicity problems. *J Bacteriol*. 2010;192(16):4089-102.
41. Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M, et al. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res*. 2005;33(17):5691-702.
42. Vogel HJB, David M. Acetylornithinase of *Escherichia coli*: Partial purification and some properties. *Journal of Biological Chemistry*. 1955;218:97-106.
43. Balch W, E.; Wolfe, R. S. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Applied and Environmental Microbiology*. 1976;32(6):781-91.

44. Norton JE, Sokatch JR. Purification and partial characterization of the branched chain amino acid transaminase of *Pseudomonas aeruginosa*. *Biochimica et biophysica acta*. 1970;206(2):261-9.
45. Ju J, Xu S, Furukawa Y, Zhang Y, Misono H, Minamino T, et al. Correlation between catalytic activity and monomer-dimer equilibrium of bacterial alanine racemases. *J Biochem*. 2011;149(1):83-9.
46. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res*. 2000;28(1):27-30.
47. Schomburg I, Chang A, Hofmann O, Ebeling C, Ehrentreich F, Schomburg D. BRENDA: a resource for enzyme data and metabolic information. *Trends in biochemical sciences*. 2002;27(1):54-6.
48. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*. 2011;7:539.

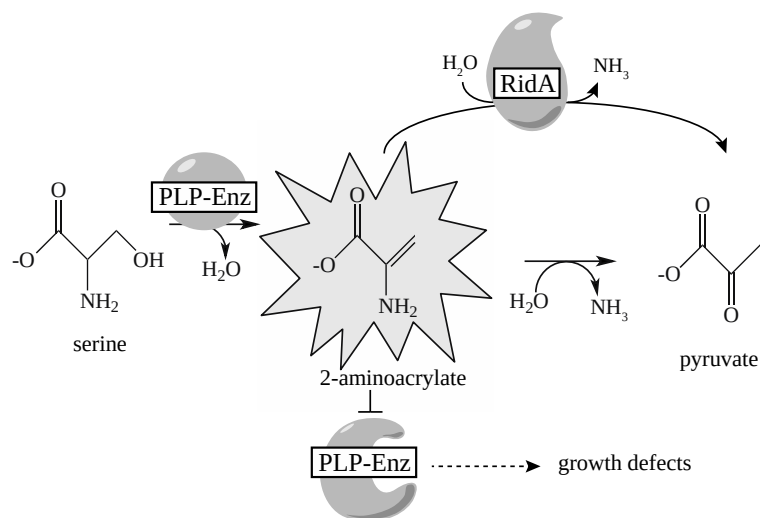


Figure 5.1 *Salmonella enterica* RidA paradigm

The serine-dependent formation of 2-aminoacrylate (2AA) by a pyridoxal 5'-phosphate enzyme (PLP-Enz) induces metabolic stress in the absence of RidA. Persisting 2AA inactivates a subset of PLP-enzymes, which leads to growth defects. 2AA is quenched by one of two routes i) spontaneous deamination by water or ii) RidA-mediated deamination.

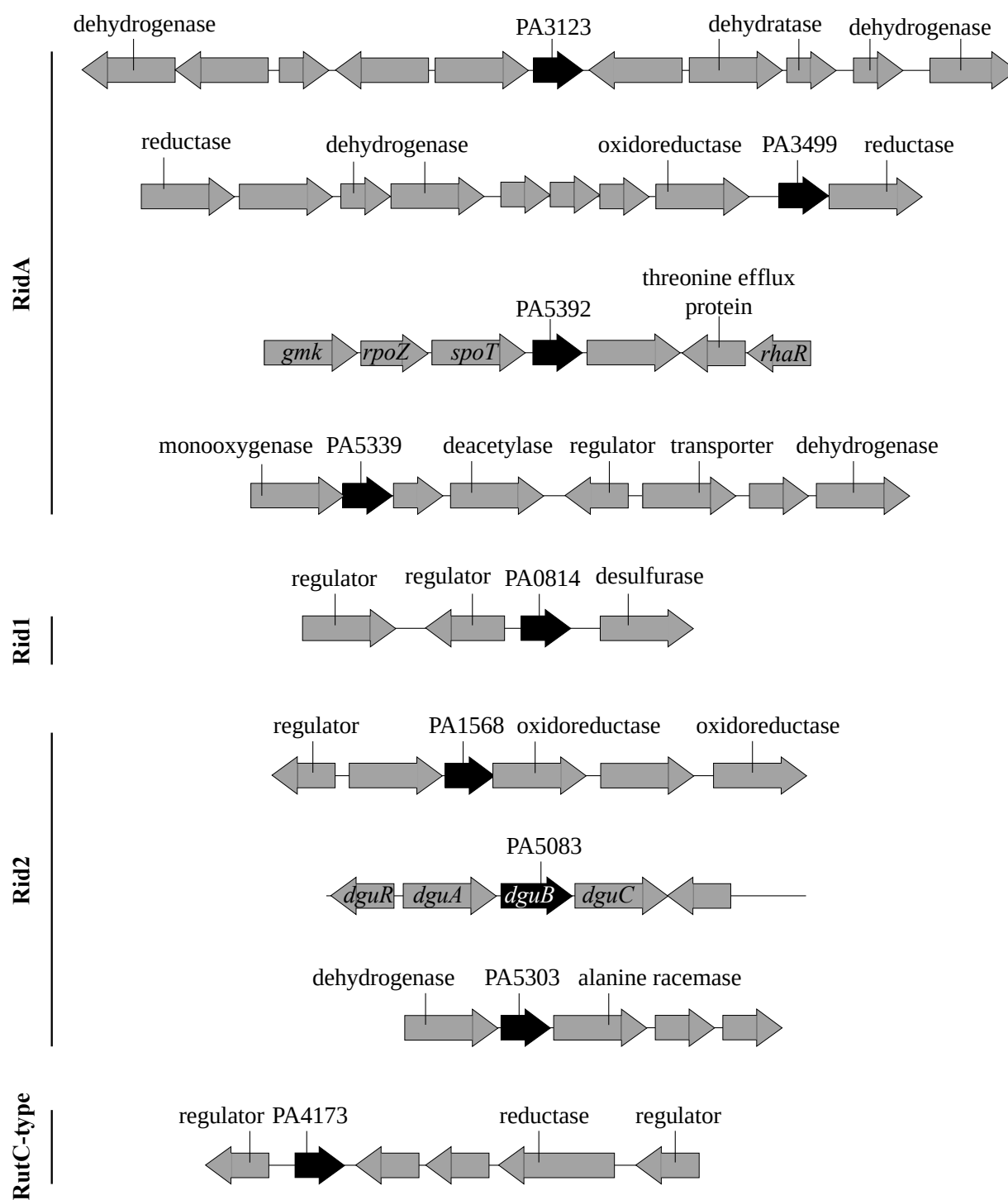


Figure 5.2 Distribution of Rid subfamily proteins in *Pseudomonas aeruginosa* PAO1
The *Pseudomonas aeruginosa* genome encodes nine members of the Rid superfamily. The subfamily assignments are listed, including PA4173 which has not been assigned a subfamily but is instead classified as a RutC-like Rid protein (40). The relative distribution of Rid proteins (black arrows) is shown along with selected annotations for putative or validated activities in close proximity to the proteins. Genome organization was evaluated using the PubSEED database (41) using the RidA subsystem originally published by Niehaus, *et al.* (6).

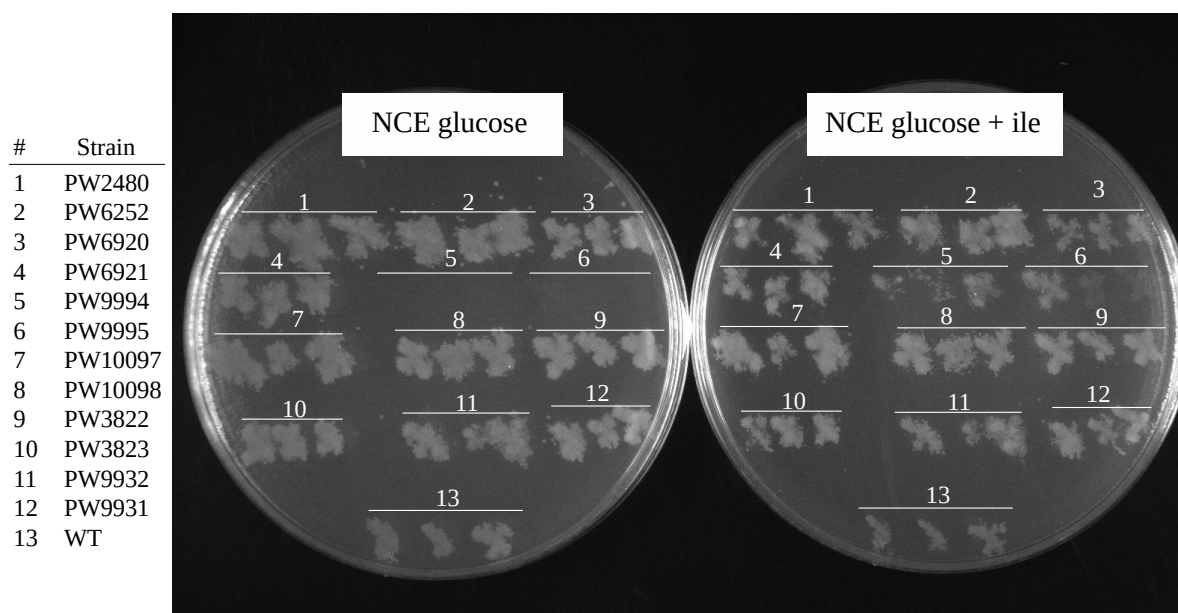


Figure 5.3 Growth of *P. aeruginosa* mutant strains on minimal medium

Pseudomonas aeruginosa strains were patched in triplicate to rich (LB) medium and printed to minimal NCE medium containing glucose and without or with isoleucine supplementation (0.3 mM). Patch plates were incubated for 3 hours at 37°C prior to printing to minimal medium. Printed plates were incubated at 37°C overnight prior to imaging.

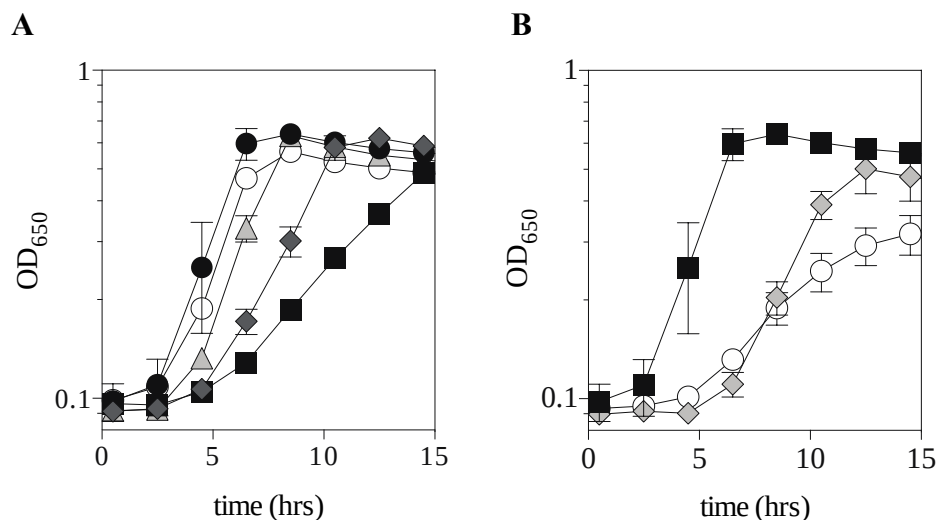


Figure 5.4 Effect of amino acid supplementation on growth of PW9994

P. aeruginosa cultures were grown in minimal NCE medium with 20 mM glucose as the sole carbon source. **(A)** Growth of the wild type (open circles) is shown without supplementation. A Δ PA5339 mutant strain (PW9994) was grown without supplementation (closed squares) or with isoleucine (closed circles), threonine (grey triangles), or glycine (grey diamonds). **(B)** A Δ PA5339 mutant strain (PW9994) was grown in minimal medium supplemented with isoleucine (closed squares), isoleucine and serine (open circles) or isoleucine and cysteine (grey diamonds). Amino acids were present at the following concentrations: isoleucine (0.3 mM), threonine (0.3 mM), glycine (1 mM), serine (5 mM), and cysteine (250 μ M). The mean of three biological replicate is plotted and error bars indicate the standard deviation.

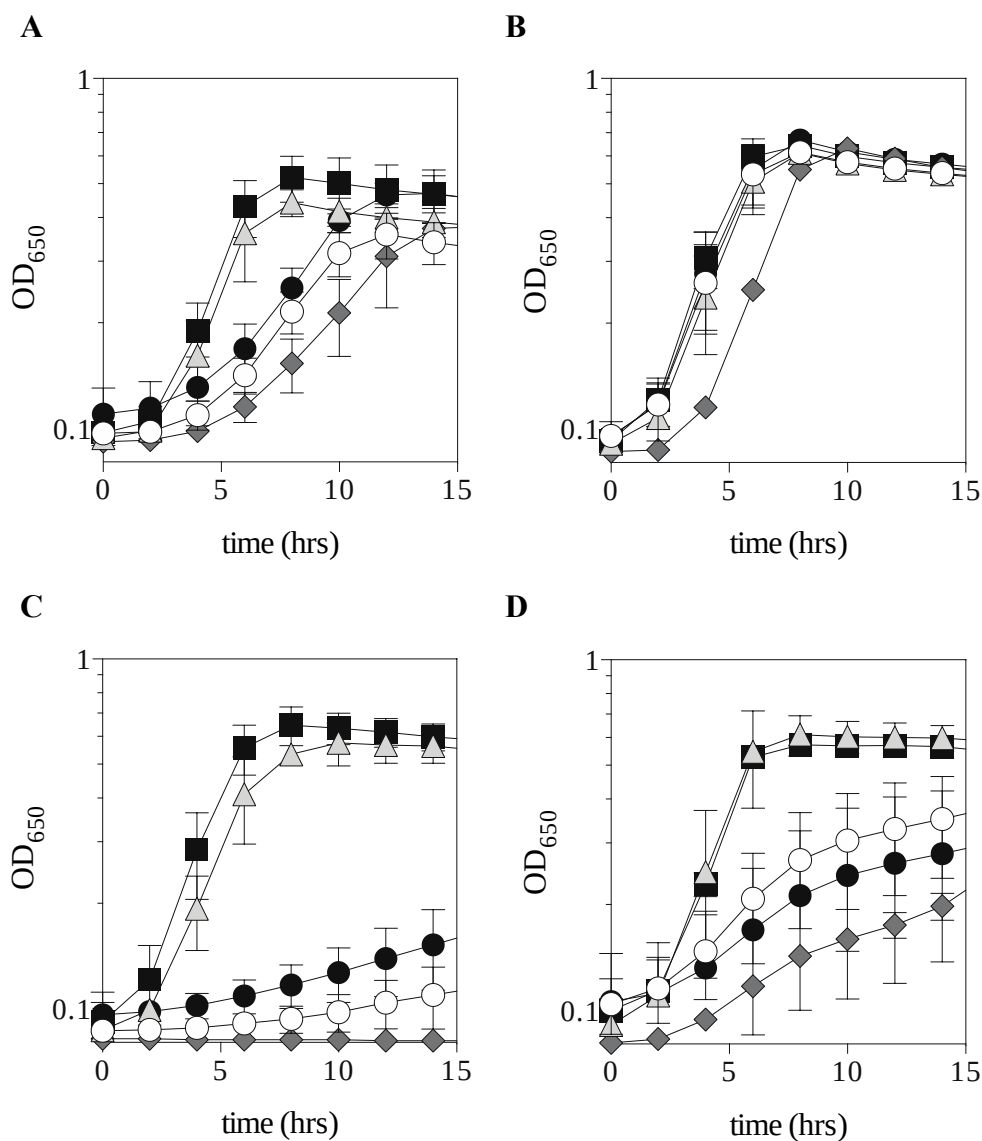


Figure 5.5 A subset of RidA proteins improve growth of *P. aeruginosa* strain PW9994
A *P. aeruginosa* Δ PA5339 mutant strain (PW9994) was transformed with pMQ72 constructs harboring cloned RidA proteins: PA5392 (grey diamonds) PA5339 (grey triangles), PA3123 (closed circles), *S. enterica* RidA (closed squares), or no insert (open circles). Cultures were grown in minimal NCE medium with 20 mM glucose as the sole carbon source and **(A)** no additional supplementation, **(B)** 0.3 mM isoleucine **(C)** 5 mM serine or **(D)** 0.3 mM isoleucine and 5 mM serine. The mean of three biological replicate is plotted and error bars indicate the standard deviation.

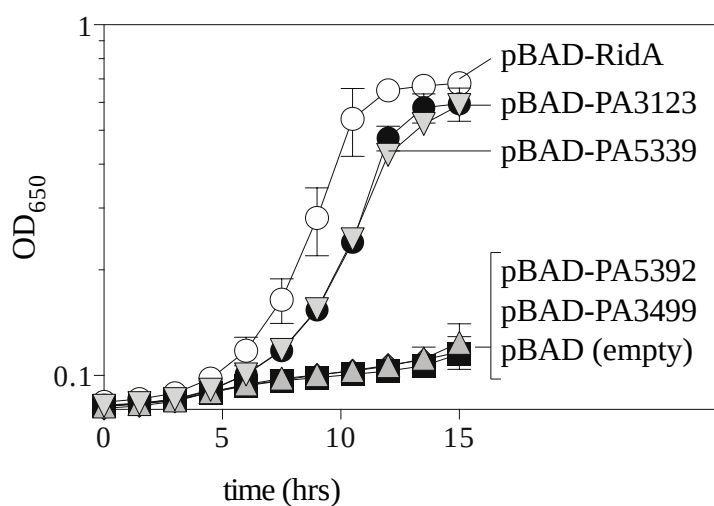


Figure 5.6 *P. aeruginosa* RidA proteins restore metabolic balance to an *S. enterica ridA* mutant strain

A *S. enterica ridA* strain (DM12920) was transformed with pBAD24 constructs. The resulting strains were grown in minimal NCE medium containing glycerol (30 mM), serine (5 mM) and arabinose (0.02%). Representative symbols for the plasmid inserts are as follows: RidA (open circles), PA3123 (closed circles), PA5339 (grey downward triangles), PA5392 (closed squares), PA3499 (grey upward triangles), and no insert (grey diamonds).

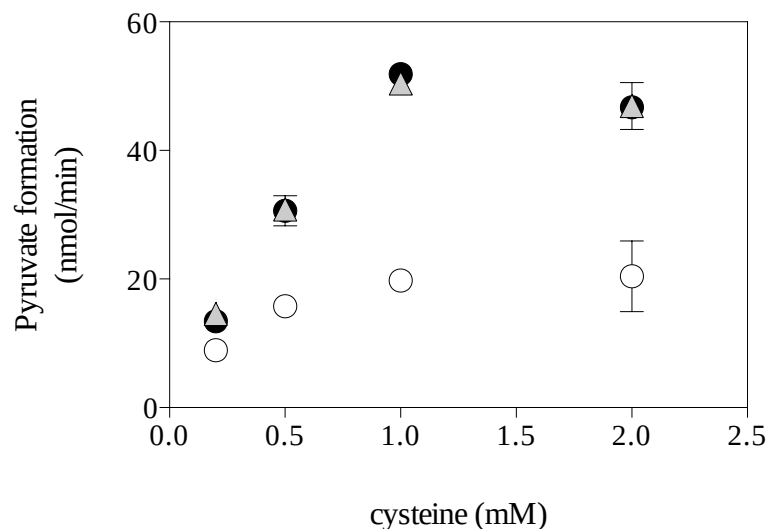


Figure 5.7 PA5339 deaminates 2AA *in vitro*

Activity assay mixtures (100 μ L) contained Tris-HCl (100 mM, pH 8), NADH (250 μ M), pyridoxal 5'-phosphate (30 μ M), pyruvate kinase/lactate dehydrogenase (5 Units), and purified cysteine desulfhydrase CdsH (0.27 μ M). RidA (closed circles) and PA5339 (grey triangles) were present at 0.19 μ M when indicated, or an equal volume of buffer was added (CdsH alone, open circles). The reaction was monitored via absorbance at 340 nm for two minutes following L-cysteine addition to the final concentrations shown. The initial rate of pyruvate formation was calculated from the rate of NADH oxidation in the first 30 seconds along with the molar extinction coefficient ($\epsilon = 6,200 \text{ M}^{-1} \text{ cm}^{-1}$). Experiments were performed in triplicate and the mean was plotted with error bars representing the standard deviation. Error bars do not appear when the error bars were shorter than the symbols used to represent the data.

Table 5.1 Strains and plasmids

Strain	Relevant Genotype	Reference
<i>P. aeruginosa</i> strains		
MPAO1	wild type	(19)
PW2480	PA0814::Tn5	(19)
PW3822	PA1568::Tn5	(19)
PW3823	PA1568::Tn5	(19)
PW6252	PA3123::Tn5	(19)
PW6920	PA3499::Tn5	(19)
PW6921	PA3499::Tn5	(19)
PW9931	PA5303::Tn5	(19)
PW9932	PA5303::Tn5	(19)
PW9994	PA5339::Tn5	(19)
PW9995	PA5339::Tn5	(19)
PW10097	PA5392::Tn5	(19)
PW10098	PA5392::Tn5	(19)
<i>S. enterica</i> strains		
DM12920	<i>ridA1</i> ::Tn10d(Tc)	(4)
DM14846	<i>ridA1</i> ::Tn10d(Tc)/pBAD24- <i>ridA</i>	
DM14847	<i>ridA1</i> ::Tn10d(Tc)/pBAD24	
DM16214	<i>ridA1</i> ::Tn10d(Tc)/pBAD24-PA5339	
DM16215	<i>ridA1</i> ::Tn10d(Tc)/pBAD24-PA5392	
DM16216	<i>ridA1</i> ::Tn10d(Tc)/pBAD24-PA3123	
DM16217	<i>ridA1</i> ::Tn10d(Tc)/pBAD24-PA3499	
<i>E. coli</i> strains		
DM16295	pDM1563 (pET28-PA5339)	
Plasmid ID	Description	Reference
pMQ72	<i>Pseudomonas</i> shuttle vector	(23)
pBAD24 (pCV1)	BspQI-modified cloning vector	(28, 29)
pDM1532	pMQ72-PA5392	This study
pDM1533	pMQ72-PA5339	This study
pDM1559	pBAD24-PA5339	This study
pDM1560	pBAD24-PA5392	This study
pDM1561	pBAD24-PA3123	This study
pDM1562	pBAD24-PA3499	This study
pDM1563	pET28-PA5339	This study
pDM1566	pMQ72-PA3123	This study
pDM1568	pMQ72- <i>ridA</i> (<i>S. enterica</i>)	This study

Table 5.2 Primers

Primer name and (Relevant strain or plasmid)	Primer sequence
PR851 (PW2480 F)	GCTGTTGGAACCGTATTCGT
PR852 (PW2480 R)	CGACAGAGGACGAGGAAGTC
PR857 (PW6252 F)	GCCATGAGCACCAACTACCT
PR858 (PW6252 R)	GTCGATATGAGCCAGGGTTG
PR859 (PW6920 F)	CAGTAGAGCAGTTCACCGA
PR860 (PW6920 R)	GGAGAACCTCTACCAGCACG
PR861 (PW6921 F)	CAGGCTATCGTGGACGAAC
PR862 (PW6921 R)	AACTTCGACTGGACCGAATG
PR863 (PW9994 F)	CGAGTACCGCATGGAAGTCT
PR864 (PW9994 R)	CCCATGATCTCGTTGACCTT
PR865 (PW9995 F)	CGAATTCAATGTCGAACTGC
PR866 (PW9995 R)	ACCTTCCAGTGCTCGTCATC
PR867 (PW10097 F)	GTTCTCGAAGGCATCCACC
PR868 (PW10097 R)	ATTCGCATGTTCAACACCAA
PR869 (PW3822 F)	GTATAGAGGGTGCCGTCCAG
PR870 (PW3822 R)	GCTGTTTCATGATGGGACTGA
PR871 (PW9932 F)	CTCATCATCACGATCTTCGC
PR872 (PW9932 R)	CCAGCACACTAACGAGCGTA
PR873 (PW9931 F)	GCGGGAGAAGTGAATCATCA
PR874 (Tn5 <i>phoA</i>)	CGGGTGCAAGTAATATCGCCCT
PR875 (Tn5 <i>lacZ</i>)	GGGTAACGCCAGGGTTTTCC
PR897 (pMQ72-PA5339)	TACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCGGTACCCGATTTCAGCAAGGAG TTCCC
PR898 (pMQ72-PA5339)	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCTTACTCGAGGACGAC GATG
PR919 (pMQ72-PA5392)	TACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCGGTACCCAGGAAGTCAGCTGAT GC
PR920 (pMQ72-PA5392)	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCTCATTGCTGCCAGTC GT
PR923 (pMQ72 seq F)	CCGCCAAAACAGCC

PR924 (pMQ72 seq R)	ACCCGTTTTTTTGGGCT
PR976 (pBAD24-PA3123)	NNGCTCTTCNTTCATGAGCGATGACATCCAGCGTTA
PR977 (pBAD24-PA3123)	NNGCTCTTCNTTAGCCCTCGCCGACCCA
PR978 (pBAD24-PA5339)	NNGCTCTTCNTTCATGACCAAGACCGTTATCCAC
PR979 (pBAD24-PA5339)	NNGCTCTTCNTTACTCGAGGACGACGATGG
PR980 (pBAD24-PA5392)	NNGCTCTTCNTTCATGCCTACCCATACCCGCATT
PR981 (pBAD24-PA5392)	NNGCTCTTCNTTATTGCTGCCAGTCGTC
PR982 (pBAD24-PA3499)	NNGCTCTTCNTTCATGACCGCGGTGC
PR983 (pBAD24-PA3499)	NNGCTCTTCNTTAGGCTTCGTCGCAG
PR1031 (pET28-PA5339)	NNGCTCTTCNATGACCAAGACCGTTATCCAC
PR1032 (pET28-PA5339)	NNGCTCTTCNGTGCTCGAGGACGACGATGG
PR1033 (pMQ72-PA3123)	CCAAGCTTG CATGCCTGCAGGTCGACTCTAGAGGATCCCCCTCAGCCCTCGCCGA CCCACGCC
PR1034 (pMQ72-PA3123)	TACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCGGTACCCCGCAGTGCTACATGA ATACGC
PR1037 (pMQ72- <i>ridA</i>)	CCAAGCTTG CATGCCTGCAGGTCGACTCTAGAGGATCCCCTTAGCGACGAACAGC GA
PR1038 (pMQ72- <i>ridA</i>)	TACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCGGTACCCCATATTGTGGTGCTGG CC

Table 5.3 Growth of selected *P. aeruginosa* strains from the transposon mutant library^a

Strain (19)	Transposon insertion locus ^b	NCE ^c	MOPS ^d	
			Glucose ^e	Succinate
MPAO1	WT	+	+	+
PW2480	PA0814	+	+	+
PW3822	PA1568	+	+	+
PW3823	PA1568	+	+	+
PW6252	PA3123	+	+	+
PW6920	PA3499	+	+	+
PW6921	PA3499	+	+	+
PW9931	PA5303	+	+	+
PW9932	PA5303	+	+	+
PW9994	PA5339	-	-	-
PW9995	PA5339	-	-	-
PW10097	PA5392	+	+	+
PW10098	PA5392	+	+	+

^a Growth was assessed on minimal medium after 24 hours at 37°C.

^b Transposon insertions were randomly generated and the location within each ORF was mapped via a combination of Tn-seq and Sanger sequencing methods (19).

^c No-carbon E medium was used as base and supplemented with MgSO₄ (42) and trace minerals (43)

^d MOPS was used as a base medium (20)

^e Glucose or succinate were added to 20 mM as the sole carbon source.

Table 5.4 *P. aeruginosa* PAO1 homologs of *S. enterica* Rida paradigm proteins^a

	<i>P. aeruginosa</i>	<i>S. enterica</i>	Percent identity ^b
2AA Generators			
Threonine dehydratase EC 4.3.1.19	IlvA1 (PA0331)	IlvA	52%
	IlvA2 (PA1326)	IlvA	54%
	PA0851	TdcB	29%
	PA2683	TdcB	38%
Cysteine desulfhydrase EC 2.5.1.47	PA1061	CdsH	59%
	PA2104		25%
	PA0932		26%
	PA2709		26%
Diaminopropionate ammonia-lyase EC 4.3.1.15	-	DpaL	-
2AA Targets			
Isoleucine transaminase EC 2.6.1.42	PA5013 (44)	IlvE	48%
Alanine racemase EC 5.1.1.1	PA4930 (45)	Alr	46%
	PA5302 (45)	DadX	48%
Serine hydroxymethyltransferase EC 2.1.2.1	GlyA1 (PA5415)	GlyA	71%
	GlyA2 (PA2444)		71%
	GlyA3 (PA4602)		75%
Aspartate aminotransferase EC 2.6.1.1	PA3139 ^c	AspC	48%

^a The information in this table was obtained from the KEGG (46) and BRENDA databases (47)

^b Percent identity was evaluated using the CLUSTAL alignment online tool (48)

^c This locus is annotated as an aromatic amino acid transaminase (EC 2.6.1.57) and is indicated as a putative aspartate aminotransferase based on sequence homology in the KEGG database.

CHAPTER 6

STRUCTURAL ANALYSIS OF RID IMINOARGININE DEAMINASE ACTIVITY¹

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6.1 ABSTRACT

The Rid (YjgF/YER057c/UK114) superfamily is conserved in all domains of life. Proteins from humans, archaea, plants and diverse bacteria share enamine/imine deaminase activity with the archetypal RidA protein from *Salmonella enterica*. Although numerous biochemical associations have been suggested between Rid proteins and various cellular processes, deaminase activity remains the best-characterized and physiologically relevant Rid activity. *In vitro*, a number of imines were identified as Rid substrates in addition to the *in vivo* stressor 2-aminoacrylate. Results from an *in vitro* reconstitution of iminoarginine production by D-amino acid dehydrogenase DauA from *Pseudomonas aeruginosa* showed iminoarginine specificity was conserved among members of the Rid2 and Rid3 subfamilies, two of the eight total Rid subfamilies. The mechanism of iminoarginine deaminase activity was probed using iminoarginine analogs and variant proteins. Together, the biochemical data are consistent with a model where the critical residue in the Rid active site is an arginine residue that binds the imine carboxyl group. Furthermore, the crystal structures of iminoarginine deaminases soaked or modeled with compounds supported this model and provided insights into iminoarginine binding.

6.2 INTRODUCTION

The Rid (YjgF/YER057c/UK114) protein superfamily is a sequence-diverse group that consists of eight subfamilies. The ubiquity of the Rid superfamily spurred a number of structure-function approaches to characterize this group. However, many crystal structures were deposited without an accompanying publication and over twenty were solved prior to the elucidation of any biochemical function for these proteins (1-8). As the list of sequenced genomes grew, it became clear that the structural data represented primarily one subfamily (RidA) out of the eight total.

This disparity prompted an effort to characterize and crystallize proteins from the Rid1-7 subfamilies, which are found only in prokaryotes.

The archetypal Rid protein, RidA from *Salmonella enterica*, is a reactive intermediate deaminase that quenches the metabolite 2-aminoacrylate (2AA), a molecule that damages certain enzymes and ultimately leads to growth defects in the absence of RidA (9-14). In addition to 2AA, a number of short-lived intermediates were substrates for RidA and other bacterial Rid proteins *in vitro* (15, 16). A biochemical study found that iminoarginine deaminase activity was unique to the Rid2 and Rid3 subfamilies (16). This substrate specificity was significant because it divided the Rid superfamily into two groups and suggested that structural features of Rid proteins that had iminoarginine deaminase activity might differ from Rid proteins that lack activity with this substrate. *In vivo*, iminoarginine is generated as an intermediate in *Pseudomonas aeruginosa* D-arginine metabolism by D-arginine dehydrogenase DauA (17-19). The Rid2 and Rid3 proteins tested (from *Pseudomonas* species) accelerated the rate of the DauA reaction *in vitro*, which led to the proposed involvement of Rid in the D-arginine utilization pathway is depicted in Figure 6.1. In addition to DauA, iminoarginine is generated from arginine by L-amino acid oxidase (EC 1.4.3.2) from *Crotalus adamanteus* (rattlesnake) (20-22), or bacterial enzymes with high specificity for arginine, citrulline, histidine and lysine (19, 23-25). Previous work has shown that the *Pseudomonas* Rid2 and Rid3 proteins tested deaminated the imine products of LOX oxidation from L-histidine, L-methionine, L-leucine, L-glutamine and L-phenylalanine *in vitro* (16).

The Rid active site is nestled between two subunits of the homotrimeric protein (5, 8). Although seven residues were proposed to be involved in catalysis, studies *in vivo* and *in vitro* with *S. enterica* RidA variants showed only residue Arg105 was required for activity (26). The

importance of the active site arginine was supported by strict conservation in the RidA, and Rid1-3 subfamilies. These observations led to the hypothesis that the arginine residue is necessary and sufficient for imine deaminase activity of any Rid protein (15). This study was initiated to evaluate which, if any, structural features were required specifically for iminoarginine deaminase activity.

A single iminoarginine deaminase crystal structure, YoaB from *S. enterica* (PDB #3GTZ), had been solved prior to 2016. Purified YoaB deaminated the imine derivatives of multiple amino acids (15), and here we report its iminoarginine deaminase activity. We compared the YoaB structure to RidA, which did not deaminate iminoarginine generated by DauA (16). A second iminoarginine deaminase structure (*P. aeruginosa* PA5083, Rid2 subfamily) was solved and the mechanistic model was refined by the results of crystal soaking experiments and molecular modeling. In order to probe the mechanism, variant YoaB and PA5083 proteins were purified and tested *in vitro*. *S. enterica* RidA was modified to generate features of Rid2 or Rid3 proteins, however, gain of iminoarginine deaminase activity was not observed. The data supported the hypothesis that the active site arginine residue was necessary and sufficient for deamination of iminoarginine, as well as 2AA. Crystal structure analyses suggested that the general capacity of the active site, rather than specific residues, might permit Rid2 and Rid3 proteins to bind iminoarginine.

6.3 MATERIALS AND METHODS

Bacterial strains, plasmids and media

Bacterial strains and plasmids used are listed in Table 6.1. *Salmonella enterica* strains listed were derivatives of Typhimurium LT2, and *Escherichia coli* BL21AI was used for protein overexpression prior to purification. Cloning and mutagenic primer sequences are provided in

Table 6.2. *S. enterica yoaB* was amplified from the chromosome and ligated between the EcoRI and HindIII restriction sites for pBAD24 or between the NdeI and XhoI sites for pET20b.

Subsequent digest of the PCR product and vector with these restriction enzymes was followed by ligation by T4 ligase (New England Biolabs) to yield pDM1386 (pBAD24-*yoaB*) and pDM1387 (pET20b-*yoaB*). A truncated allele (*ridA635*) was generated via amplification of the *ridA* locus with primers that included a BspQI restriction site and subsequent cloning into BspQI-modified vector derivatives of pET28 (27) or pBAD4 (28). Additional plasmids that harbor Rid proteins were cloned using BspQI sites, and the relevant plasmids have been published (16). Site-directed mutagenesis was done via amplification with mutagenic primers followed by DpnI digest and transformation according to the Quikchange II Site-Directed Mutagenesis protocol (Agilent). Plasmid insert sequences were verified by sequencing performed by Genewiz (South Plainfield, NJ), Eton Biosciences (San Diego, CA), or the Georgia Genomics Facility (Athens, GA).

Difco Nutrient Broth (8 g/L) with NaCl (5g/L) served as a rich medium for *S. enterica* and Lysogeny broth (LB) was used for *E. coli* overnight cultures. Strains were grown in superbroth (32 g/L tryptone, 20 g/L yeast extract, 5 g/L NaCl and 0.2 g/L NaOH) for protein overexpression. Minimal medium contained no-carbon E salts (NCE) supplemented with MgSO₄ (1 mM) (29), trace minerals (30) and glycerol (30 mM). Serine (5 mM) and cysteine (250 μM) were added to the growth medium when indicated. For plasmid maintenance, antibiotics were added at the following concentrations: ampicillin, 150 mg/L; kanamycin, 50 mg/L. Chemicals were purchased from Millipore Sigma (Sigma Aldrich, St. Louis, MO). Crystalline L-amino acid oxidase (A9253 Type I, dried venom *Crotalus adamanteus*) and catalase from bovine liver (C9322) were hydrated and stored kept on ice until use in biochemical assays.

Growth analysis

Growth analyses in liquid medium were done in a 96-well microtiter dish using a BioTek Elx808 plate reader set to slow shaking speed. *S. enterica* strains were grown in biological triplicate overnight in NB medium (2 mL) and were used to inoculate minimal glycerol medium (1% inoculum). Growth was monitored via optical density (OD) at 650 nm at 37°C and growth curves were plotted using GraphPad Prism (version 6.0g).

Protein purification

Polyhistidine-tagged Rid proteins were purified from *E. coli* BL21AI harboring the appropriate pET construct using nickel affinity chromatography. The protein purification protocol was based on the purification schemes for RidA (31) and several Rid proteins (PAO814 and PA5083 from *Pseudomonas aeruginosa* PAO1, PFL_1385 from *Pseudomonas fluorescens* Pf-5, PSPTO_3006 and PSPTO_0102 from *Pseudomonas syringae* DC3000 and ACIAD3089 from *Acinetobacter baylyi* ADP1) (16). Induction time and incubation temperature modifications were made to maximize protein recovery. Briefly, YoaB proteins, RidA variants, and PA5083^{R93A} were purified as follows. An overnight culture (LB, 10 mL) was used to inoculate a flask containing 1.5 liters of superbroth supplemented with the appropriate drug. A total of 3 liters was inoculated for YoaB wild type, YoaB^{W12Y}, YoaB^{Q45N}, RidA^{R105A}, and RidA^{ΔSer2-Gly15} to increase biomass. Cultures were grown at 37°C until they reached an OD₆₅₀ between 0.3 and 1.0 and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 1mM) and arabinose (0.2-0.2%) and shifted to a lower temperature for an additional 18 hours. The induction conditions for each protein were determined SDS-PAGE gel analysis and the OD₆₅₀ at induction, arabinose concentration, and post-induction temperatures were as follows: YoaB wild type (OD = 0.7, 0.2% arabinose, 28°C), YoaB^{W12Y} (OD = 0.5, 0.2% arabinose, 28°C), YoaB^{R92A} (OD = 1.0, 0.2%

arabinose, 22°C), YoaB^{Q45N} (OD = 0.5, 0.2% arabinose, 28°C), YoaB^{Q45A} (OD = 1.0, 0.2% arabinose, 22°C), YoaB^{Q45D} (OD = 1.0, 0.2% arabinose, 22°C), YoaB^{Q45E} (OD = 1.0, 0.2% arabinose, 22°C), YoaB^{Q45L} (OD = 1.0, 0.2% arabinose, 22°C), RidA^{ΔSer2-Gly15} (OD = 1.0, 0.2% arabinose, 28°C), RidA^{R105A} (OD = 0.7, 0.2% arabinose, 28°C), RidA^{Y17W} (OD = 0.3, 0.02% arabinose, 22°C), RidA^{Y17M} (OD = 0.3, 0.2% arabinose, 22°C), RidA^{Y17L} (OD = 0.3, 0.2% arabinose, 22°C), PA5083^{R93A} (OD = 0.3, 0.02% arabinose, 22°C). The cell pellets were harvested via centrifugation (7,000 x g) and the pellets were stored at -80°C until use. Binding buffer (50 mM potassium phosphate, pH 7.5; 100 mM NaCl; 5 mM imidazole; and 10% glycerol) was added to thaw the cells (2 mL per gram wet cell weight). Cells were lysed in a French pressure cell, the lysate was clarified via centrifugation (40,000 x g) and passed through a syringe filter (0.45 micron, Argos Technologies) and loaded onto a nickel column. YoaB wild type, YoaB^{W12Y}, YoaB^{Q45N}, RidA^{ΔSer2-Gly15}, and RidA^{R105A} were purified using an ÄKTA FPLC equipped with a column containing HisPur Ni-NTA Superflow resin (5 mL, ThermoFisher Scientific) and the remaining proteins were loaded onto freshly poured gravity columns (1 mL HisPur resin) to limit any contamination that might occur from previous protein purifications. Protein was eluted over a gradient (0-100%) of elution buffer (50 mM potassium phosphate, pH 7.5; 100 mM NaCl; 0.5 M imidazole; and 10% glycerol). Elution of the proteins from the FPLC column was monitored using a UV lamp at 280 nm. Fractions from both purification methods containing the desired protein were identified using SDS-PAGE analysis, and the protein was dialyzed into storage buffer (50 mM potassium phosphate, pH 7.5 and 10% glycerol) using a PD-10 desalting column (GE Healthcare). Purified proteins were drop-frozen in liquid nitrogen and stored at -80°C. Concentrations were determined by BCA protein assay (ThermoScientific).

Enzyme assays

Rid protein activity was assayed at room temperature using L-amino acid oxidase in a continuous assay as described previously (15, 16, 32) using a quartz microtiter plate and a Spectramax Plus384 (Molecular Devices, Sunnyvale, CA). The reaction mixture (100 μ L) contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), bovine liver catalase (24 Units), L-amino acid oxidase from *Crotalus adamanteus* (0.4 μ M), and Rid proteins (10 μ M). Reactions were initiated with the addition of L-amino acid or 2-aminobutyrate to the indicated concentration. Immediately following substrate addition and mixing (5 seconds), the path length was measured. The absorbance at 248 nm was monitored for five minutes and the slope, path length, and molar extinction coefficient for semicarbazone ($\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$) were used to calculate the rate of semicarbazone formation. A modification was made to the assay to test iminopropionate deaminase activity and D-amino acid oxidase from porcine kidney (10 milli-Units) was added in place of L-amino acid oxidase. The reaction was initiated with D-alanine (10 mM) and the rate of semicarbazone formation was determined in the same manner as reactions containing LOX.

Alignments

Molecular structure analyses were performed with the UCSF Chimera package (v 1.11.2, Build 41376) (33). Structures were aligned with Chimera using the Needleman-Wunsch algorithm and amino acid side chains were queried using the Dunbrack rotamer library (34). Amino acid sequence alignments were generated using the MUSCLE alignment settings with Geneious (v 8.1.9, Build 2016-09-27) using a non-commercial floating R10 license to UGA.

6.4 RESULTS AND DISCUSSION

YoaB is an iminoarginine deaminase

The *S. enterica* YoaB crystal structure (PDB #3GTZ) was deposited in 2009, prior to the elucidation of the RidA paradigm. Purified YoaB (Rid2 subfamily) deaminated imine intermediates from leucine, phenylalanine, methionine and glutamine, and was validated as a deaminase as predicted by the presence of Arg92 (15). YoaB activity was tested using a standard imine deaminase assay with L-amino acid oxidase (LOX) and L-arginine (15) and the results are shown in Table 6.3. YoaB decreased the rate of semicarbazone formation, indicating it had iminoarginine deaminase activity. These results suggested the YoaB structure might be a suitable Rid2 representative in an initial comparison to non-iminoarginine deaminase structures.

Deamination of arginine analogs

Arginine analogs were used to probe the Rid2/Rid3 specificity using a biochemical activity assay in which imines were produced *in situ* via LOX oxidation of citrulline or ornithine. The expected oxidation products of these amino acids were chemically similar to iminoarginine, but differed at particular functional groups. Any correlation between functional groups and Rid activity, might suggest ways to probe a mechanistic model. We predict that the interaction between the Rid protein arginine residue and the imine carboxyl group is necessary and sufficient for binding (15). LOX did not deaminate L-arginine methyl ester so it was not possible to test whether methylating the imine carboxyl group would abolish Rid activity. Eight purified proteins (*S. enterica* RidA, *Pseudomonas aeruginosa* PA0814, *Acinetobacter baylyi* ADP3089, *S. enterica* YoaB, *Pseudomonas syringae* PSPTO_0102, *P. aeruginosa* PA5083, *Pseudomonas fluorescens* PFL_1385, and *P. syringae* PSPTO_3006) were screened for activity on the imine derivatives of citrulline or ornithine. Figure 6.2 shows activity of each protein with

iminoarginine and iminocitrulline. When ornithine was used as a LOX substrate, the effect of Rid proteins on the rate was small and consistent across the subfamilies (data not shown). Ornithine and citrulline are similar in size to arginine but have modified guanidinium moieties. Ornithine can undergo spontaneous lactamization (35). If the imine derivative of ornithine undergoes a similar change, it might prevent entry to the Rid active site and could explain the overall poor Rid deaminase activity. Preliminary studies with *S. enterica* RidA showed activity on the imine derived from canavanine as well (data not shown). Together, these data supported the designation for Rid proteins as broad *in vitro* deaminases and identified iminocitrulline as an additional substrate. As the Rid role(s) in metabolism are elucidated, any enzymes that oxidize citrulline or canavanine should be investigated as possible *in vivo* imine sources.

Considerations regarding iminoarginine binding

An identical iminoarginine product is expected to result from enzymatic oxidation of either arginine stereoisomer (18, 36). However, two pieces of evidence suggested there might be subtle differences between the molecules generated. First, LOX-derived iminoarginine seemed to serve as a substrate for RidA and Rid1 proteins (Figure 6.2A), in contrast to DauA-derived iminoarginine (16). Second, YoaB deaminated iminoarginine produced by LOX (Figure 6.2A) but not DauA (data not shown). These observations suggested that the stereochemistry of the amino acid precursor might influence imine chemistry. This observation, in combination with active site measurements of the PA5083 crystal structure, led to the hypothesis that the imine intermediates may assume different rotamer conformations when bound.

Figure 6.3 shows a structural alignment of Rid2 protein *S. enterica* YoaB with *S. enterica* RidA (Figure 6.3A), including a superimposition of the active site arginine residues (Figure 6.3B). This overlay showed that the two proteins shared similar active site clefts and retained

nearly identical positioning of the arginine, yet had differences in the loop features that surround the active site. In particular, YoaB seemed to have a reduced substrate binding loop (YoaB amino acids 6-14) compared to RidA (amino acids 1-15), and lacked the secondary loop (RidA amino acids 36-46). Ketoacids 2-ketobutyrate and pyruvate were soaked into the PA5083 crystal active site, and the distance was measured between the Arg93 guanidinium moiety and the substrate carboxyl group (Figure 6.3C-D). Both compounds were within possible hydrogen bonding range (~1.5 to 2.9) with Arg93, which supported the idea that this was the critical interaction for Rid protein binding. However, it seemed possible that alterations to the arginine side chain bond angles might decrease this distance, and the Dunbrack rotamer library was queried to identify plausible conformations for PA5083 Arg93 (34). This function revealed multiple possibilities that would alter the distance between the guanidinium moiety and the substrate carboxyl group. An example of an Arg93 rotamer with decreased bond distance to each substrate is shown in Figure 6.3E-F. These observations are consistent with a model where substrate interaction with the active site arginine residue is critical for binding.

In the same vein, it seemed reasonable that rotamer conformation of the bound substrate might also influence dynamics of the Rid active site when faced with larger molecules such as iminoarginine. For instance, Glu108 may prevent iminoarginine from binding in the canonical conformation, and might alter bond angles of the molecule distal to the bound carboxylic acid moiety. It is difficult to capture a clear conformational picture of the relevant Rid substrate due to the short imine half-lives, and it was not possible to suggest which rotamer might best reflect bound iminoarginine based on the biochemical data.

A few points were taken from experiments soaking the crystals with 2-ketobutyrate and pyruvate. First, this work supported the seminal studies done by Burman, *et al.*, studying the

TdcF active site with bound 2-ketobutyrate (8). As crystal structures deposited in the protein data bank are reevaluated for ligand relevance, this work supports efforts to obtain structural data with physiologically relevant ligands. Second, the fact that a ketoacid bound within the Rid active site was surprising, given that ketoacids are the products of Rid deamination and reaction products are not traditionally candidates for soaking experiments. However, this observation was consistent with a scenario where the active site arginine might bind any molecule with a compatible carboxylic acid, such as an amino acid, imine, enamine, or a ketoacid. Attempts to soak amino acids into the active site were not successful. As it has not been possible to provide short-lived enamine/imine intermediates as soaking ligands, we propose stable ketoacids to be informative ligands and suggest that these studies will serve as a model for future Rid crystallography endeavors. Further understanding of the Rid mechanism might come from understanding the dissociation constant of the Arg93-ketoacid interaction.

YoaB comparison to RidA indicates a distinct cellular role

YoaB did not demonstrate biologically relevant 2AA deaminase activity *in vivo* or *in vitro*, despite its activity on multiple imines *in vitro*. *S. enterica ridA* mutants have severe growth defects despite the chromosomal *yoaB* locus. A plasmid harboring *yoaB* (pDM1386) failed to complement a *S. enterica ridA* strain, even with induction via arabinose addition (data not shown). It was possible that YoaB was unstable or not active *in vivo*, however it seemed unlikely, as we were able to purify active protein that deaminated multiple imines derived from LOX. Purified YoaB was tested for deaminase activity using standard RidA biochemical assays using threonine dehydratase (IlvA EC 4.3.1.19) (26) or cysteine desulfhydrase (CdsH EC 4.4.1.15) (37) in continuous assays or in a competition assay with ferricyanide. In each assay under conditions where RidA was active, YoaB activity was not detected (data not shown),

suggesting YoaB did not deaminate 2AA or 2-aminocrotonate (2AC). This point is significant because 2AA and 2AC are the only Rid substrates that have demonstrated physiological roles. Therefore, YoaB does not fit into the current RidA model as a 2AA stress response protein and likely performs a separate cellular role in *S. enterica*. *In vitro* YoaB activity in other contexts suggests a role elsewhere in metabolism.

The *S. enterica* genome lacks any LOX or DauA homologs, which might generate iminoarginine, but at least two possible imine sources exist including D-amino acid dehydrogenase DadA (EC 1.4.99.1) and L-aspartate oxidase NadB (EC 1.4.3.16), which catalyzes the first step in de novo NAD⁺ biosynthesis in prokaryotes (Tedeschi, 2010). Future studies should address the *in vivo* and *in vitro* connections between these areas of metabolism and YoaB. In general, the *in vivo* role(s) of Rid proteins should be considered paramount, and the biochemical activities should inform testable genetic hypotheses. As the number of sequenced genomes increases, the pressure to annotate genes based on sequence similarity rises. However, it is likely true that many Rid1-7 proteins, whose functional roles that have yet to be defined, will perform functions distinct from characterized RidA proteins.

Probing the residues involved in iminoarginine deaminase activity.

The residues required for iminoarginine deaminase activity were probed using strategies to identify i) gain of function RidA variants or ii) YoaB variants that lost activity only on iminoarginine. Finally, we tested whether the RidA substrate-binding loop was required for activity by constructing an N-terminal truncation. The effects of these residue changes on activity are described here.

RidA variants did not gain iminoarginine deaminase activity

Purified RidA and Rid1 proteins had no observable iminoarginine deaminase activity in an assay with DauA (16). *S. enterica* RidA had some iminoarginine deaminase activity in an assay with LOX (Figure 6.2). Based on these observations it seemed likely that there might be structural differences between the iminoarginine deaminases and non-iminoarginine deaminases, such as a critical binding residue or an active site capacity that could accommodate the 5-carbon intermediate. In order to probe relevant residues, variant proteins were designed, purified and tested *in vitro* for improved iminoarginine deaminase activity. Candidate residues were selected based on analysis of an amino acid alignment of multiple RidA, Rid1-3 proteins shown in Figure 6.4.

The alignment showed the residue analogous to RidA Tyr17 was strictly conserved in RidA and Rid1 proteins. In contrast, Rid3 proteins had isoleucine at this position, and the residue was variable in Rid2 proteins (tryptophan, leucine, or methionine). It was reasonable to suggest this residue might contribute to substrate specificity. This question was probed by replacing RidA Tyr17 with leucine, methionine or tryptophan. The resulting variant proteins were assessed for any gain of iminoarginine deaminase activity, however there was no statistically significant difference compared to wild type RidA (Figure 6.5A). RidA^{Y17W}, RidA^{Y17M}, and RidA^{Y17L} deaminated iminobutyrate significantly more than the RidA^{R105A} variant, which indicated that the Tyr17 substitution proteins were active (Figure 6.5B). These results supported the hypothesis that Arg105 is necessary and sufficient, and suggested that modifications to the RidA tyrosine residue alone cannot confer iminoarginine specificity. In a complementary experiment, we tested the activity of YoaB variant proteins designed to mimic RidA.

Residues that influenced YoaB activity

Three YoaB residues were modified in order to test whether they were required specifically for iminoarginine deaminase activity. The residues were selected from the seven invariant residues proposed to comprise the active site (5, 8) and were each modified to mimic the RidA active site. If a single residue conferred iminoarginine deaminase activity, we predicted variant proteins could be identified that lacked activity solely on iminoarginine. However, the YoaB modifications described here seemed to affect activity on multiple substrates.

Alanine substitutions at Arg92 or Trp12 diminished YoaB activity on both iminoarginine and iminopropionate (Figure 6.5), which suggested that these residues were universally required. Although this was expected for Arg92, the null variant provided an important control that indicated the effect of YoaB on the rate of semicarbazone formation was due to activity of the protein. The influence of Trp12 on activity was surprising. Combined with the RidA^{Y17W} variant activity, it is possible that Trp12 is necessary but not sufficient for iminoarginine deaminase activity. This seemed contradictory given that active iminoarginine deaminases have been isolated that had methionine or leucine residues at this position (16). YoaB variant protein stability was not investigated, so it is also possible that these changes affected the general protein structure and led to decreased activity. Without an *in vivo* role for YoaB, it is difficult to interpret the physiological relevance of the active site tryptophan residue.

Changes to Gln45 affected YoaB activity differently when comparing activity on iminoarginine or iminopropionate. Introducing asparagine, alanine, aspartate or glutamate at this residue reduced, but did not completely abolish, activity on iminoarginine (Figure 6.5A). However, changing this residue to leucine completely abolished activity on both iminoarginine and iminopropionate. We hypothesized that changing the YoaB active site to mimic RidA might

shift the substrate profile toward the affinities observed for RidA, but this was not the case. Given that these changes were subtle and no *in vivo* role for YoaB exists, these variant protein studies should serve as a foundation for analysis of a Rid2 protein with an *in vivo* context, such as *Campylobacter jejuni* CfiQ (Cj0327), which plays a role in agglutination and virulence in the *Galleria mellonella* model (38).

The RidA substrate binding loop is dispensable

Crystal soaking experiments suggested that RidA homolog *Escherichia coli* TdcF might undergo a conformational change with substrate bound. The substrate-binding loop (amino acids Glu8-Val18) that connects β -sheets $\beta 1$ and $\beta 2$ (1) moved the greatest distance when 2-ketobutyrate was bound (8). In order to probe the involvement of the loop in catalysis, a variant protein with an N-terminal truncation was designed and tested for activity *in vivo* and *in vitro*. Expression of pDM1471 (pBAD24-*ridA*635), which encoded the truncated protein, rescued the growth defects of a *S. enterica ridA* mutant strain (Figure 6.6). The truncated protein was also purified without solubility issues, but was less active than the wild type RidA protein in LOX assays with multiple substrates (Table 6.4). 2AA deaminase activity was not detected when purified RidA^{Ser2-Gly15} was tested in a standard assay using CdsH (data not shown), which was inconsistent with the complementation data. It is possible that the protein remains soluble but denatures or misfolds *in vitro*, and might account for the decreased activity. Due to its unaffected activity *in vivo*, any possible misfolding of the RidA^{Ser2-Gly15} variant was not pursued, and it seemed most likely that the substrate binding loop was indispensable *in vivo*.

The active site arginine residue is required for iminoarginine deaminase activity

Alanine substitutions to the active site arginine residue abolished activity of two Rid2 proteins (YoaB^{R92A} and PA5083^{R93A}) on iminoarginine and iminobutyrate. Figure 6.7 shows PA5083 wild type activity and PA5083^{R93A} loss of function on iminoarginine and iminobutyrate, indicating that Arg93 is necessary for activity. The data are consistent with the current model that an arginine residue in this position is required to bind the carboxyl group of all Rid substrates.

Conclusions

The structural data in combination with variant protein activity support a model in which the RidA active site arginine residue binds a reactive metabolite via the carboxyl group, bringing the substrate close to water molecule(s). While other enzyme-substrate interactions likely take place and might influence Rid activity, the active site arginine residue is critical. Importantly, this model predicts that product release will therefore be slow after a reactive intermediate is deaminated by water. We propose that the arginine-carboxyl interaction will dictate the binding and release of any Rid substrate. Furthermore, because Rid amino acid backbones are proposed to participate in the mechanism by binding water molecules, it seemed likely that many functional groups could then be present within the active site without affecting activity. This scenario is supported by the sequence diversity that exists among members of the Rid family. The generation of numerous amino acid changes in YoaB with little effect on activity also supports this scenario. With the current biochemical data, it was not possible to specify the conformation or tautomer state of bound substrate. Taken together, these findings support a model where the Rid active site arginine binds various imine substrates through interaction with the carboxylic acid.

6.5 ACKNOWLEDGMENTS

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6.6 REFERENCES

1. Volz K. A test case for structure-based functional assignment: the 1.2 Å crystal structure of the yjgF gene product from *Escherichia coli*. *Protein science : a publication of the Protein Society*. 1999;8(11):2428-37.
2. Sinha S, Rappu P, Lange SC, Mantsala P, Zalkin H, Smith JL. Crystal structure of *Bacillus subtilis* YabJ, a purine regulatory protein and member of the highly conserved YjgF family. *Proc Natl Acad Sci U S A*. 1999;96(23):13074-9.
3. Deaconescu AM, Roll-Mecak A, Bonanno JB, Gerchman SE, Kycia H, Studier FW, et al. X-ray structure of *Saccharomyces cerevisiae* homologous mitochondrial matrix factor 1 (Hmf1). *Proteins*. 2002;48(2):431-6.
4. Deriu D, Briand C, Mistiniene E, Naktinis V, Grutter MG. Structure and oligomeric state of the mammalian tumour-associated antigen UK114. *Acta crystallographica Section D, Biological crystallography*. 2003;59(Pt 9):1676-8.
5. Parsons L, Bonander N, Eisenstein E, Gilson M, Kairys V, Orban J. Solution structure and functional ligand screening of HI0719, a highly conserved protein from bacteria to humans in the YjgF/YER057c/UK114 family. *Biochemistry*. 2003;42(1):80-9.
6. Manjasetty BA, Delbruck H, Pham DT, Mueller U, Fieber-Erdmann M, Scheich C, et al. Crystal structure of *Homo sapiens* protein hp14.5. *Proteins*. 2004;54(4):797-800.
7. Miyakawa T, Lee WC, Hatano K, Kato Y, Sawano Y, Miyazono K, et al. Crystal structure of the YjgF/YER057c/UK114 family protein from the hyperthermophilic archaeon *Sulfolobus tokodaii* strain 7. *Proteins*. 2006;62(2):557-61.
8. Burman JD, Stevenson CE, Sawers RG, Lawson DM. The crystal structure of *Escherichia coli* TdcF, a member of the highly conserved YjgF/YER057c/UK114 family. *BMC Struct Biol*. 2007;7:30.
9. Enos-Berlage JL, Langendorf MJ, Downs DM. Complex metabolic phenotypes caused by a mutation in yjgF, encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of bacteriology*. 1998;180(24):6519-28.
10. Schmitz G, Downs DM. 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. *Journal of bacteriology*. 2004;186(3):803-10.

11. Flynn JM, Christopherson MR, Downs DM. Decreased coenzyme A levels in ridA mutant strains of *Salmonella enterica* result from inactivated serine hydroxymethyltransferase. *Mol Microbiol.* 2013;89(4):751-9.
12. Flynn JM, Downs DM. In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5'-phosphate cofactor. *J Bacteriol.* 2013;195(16):3603-9.
13. Lambrecht JA, Schmitz GE, Downs DM. RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life. *MBio.* 2013;4(1):e00033-13.
14. Downs DM, Ernst DC. From microbiology to cancer biology: the Rid protein family prevents cellular damage caused by endogenously generated reactive nitrogen species. *Molecular microbiology.* 2015;96(2):211-9.
15. Niehaus TD, Gerdes S, Hodge-Hanson K, Zhukov A, Cooper AJ, ElBadawi-Sidhu M, et al. Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC Genomics.* 2015;16:382.
16. Hodge-Hanson KM, Downs DM. 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.* 2017;12(9):e0185544.
17. Demidkina TV, Anston AA, Faleev NG, Phillips RS, Zakomyrdina LN. Spatial structure and mechanism of tyrosine phenol-lyase and tryptophan indole-lyase. *Molekuliarnaia biologii.* 2009;43(2):295-308.
18. Yuan H, Fu G, Brooks PT, Weber I, Gadda G. Steady-state kinetic mechanism and reductive half-reaction of D-arginine dehydrogenase from *Pseudomonas aeruginosa*. *Biochemistry.* 2010;49(44):9542-50.
19. Isobe K, Sugawara A, Domon H, Fukuta Y, Asano Y. Purification and characterization of an L-amino acid oxidase from *Pseudomonas* sp. AIU 813. *J Biosci Bioeng.* 2012;114(3):257-61.
20. Wellner D, Meister A. Crystalline L-amino acid oxidase of *Crotalus adamanteus*. *J Biol Chem.* 1960;235:2013-8.
21. Jann AM, Hideki; Haas, Dieter. The fourth arginine catabolic pathway of *Pseudomonas aeruginosa*. *Journal of general microbiology.* 1988;134:1043-53.
22. Massey V, Curti B. On the reaction mechanism of *Crotalus adamanteus* L-amino acid oxidase. *J Biol Chem.* 1967;242(6):1259-64.
23. Duerre JA, Chakrabarty S. l-amino acid oxidases of *Proteus rettgeri*. *J Bacteriol.* 1975;121(2):656-63.

24. Geueke B, Hummel W. A new bacterial L-amino acid oxidase with a broad substrate specificity: purification and characterization. *Journal of Enzyme and Microbial Technology*. 2002;31:77-87.
25. Gau AE, Heindl A, Nodop A, Kahmann U, Pistorius EK. L-amino acid oxidases with specificity for basic L-amino acids in cyanobacteria. *Z Naturforsch C*. 2007;62(3-4):273-84.
26. Lambrecht JA, Flynn JM, Downs DM. Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions. *J Biol Chem*. 2012;287(5):3454-61.
27. Galloway NR, Toutkoushian H, Nune M, Bose N, Momany C. Rapid Cloning For Protein Crystallography Using Type IIS Restriction Enzymes. *Crystal Growth & Design*. 2013;13(7):2833-9.
28. VanDrise CM, Escalante-Semerena JC. New high-cloning-efficiency vectors for complementation studies and recombinant protein overproduction in *Escherichia coli* and *Salmonella enterica*. *Plasmid*. 2016;86:1-6.
29. Vogel HJB, David M. Acetylornithinase of *Escherichia coli*: Partial purification and some properties. *Journal of Biological Chemistry*. 1955;218:97-106.
30. Balch W, E.; Wolfe, R. S. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Applied and Environmental Microbiology*. 1976;32(6):781-91.
31. Lambrecht JA, Browne BA, Downs DM. Members of the YjgF/YER057c/UK114 family of proteins inhibit phosphoribosylamine synthesis in vitro. *J Biol Chem*. 2010;285(45):34401-7.
32. Hafner EWW, Daniel. Reactivity of the imino acids formed in the amino acid oxidase reaction. *Biochemistry*. 1979;18(3):411-7.
33. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem*. 2004;25(13):1605-12.
34. Dunbrack RL, Jr. Rotamer libraries in the 21st century. *Current opinion in structural biology*. 2002;12(4):431-40.
35. Murray K, Rasmussen PS, Neustaedter J, Luck JM. The Hydrolysis of Arginine. *The Journal of biological chemistry*. 1965;240:705-9.
36. Li C, Lu CD. Arginine racemization by coupled catabolic and anabolic dehydrogenases. *Proc Natl Acad Sci U S A*. 2009;106(3):906-11.

37. Ernst DC, Lambrecht JA, Schomer RA, Downs DM. Endogenous synthesis of 2-aminoacrylate contributes to cysteine sensitivity in *Salmonella enterica*. *Journal of bacteriology*. 2014;196(18):3335-42.
38. Reuter M, Periago PM, Mulholland F, Brown HL, van Vliet AH. A PAS domain-containing regulator controls flagella-flagella interactions in *Campylobacter jejuni*. *Front Microbiol*. 2015;6:770.
39. Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M, et al. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res*. 2005;33(17):5691-702.
40. Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol*. 1995;177(14):4121-30.
41. Way JC, Davis MA, Morisato D, Roberts DE, Kleckner N. New Tn10 derivatives for transposon mutagenesis and for construction of lacZ operon fusions by transposition. *Gene*. 1984;32(3):369-79.

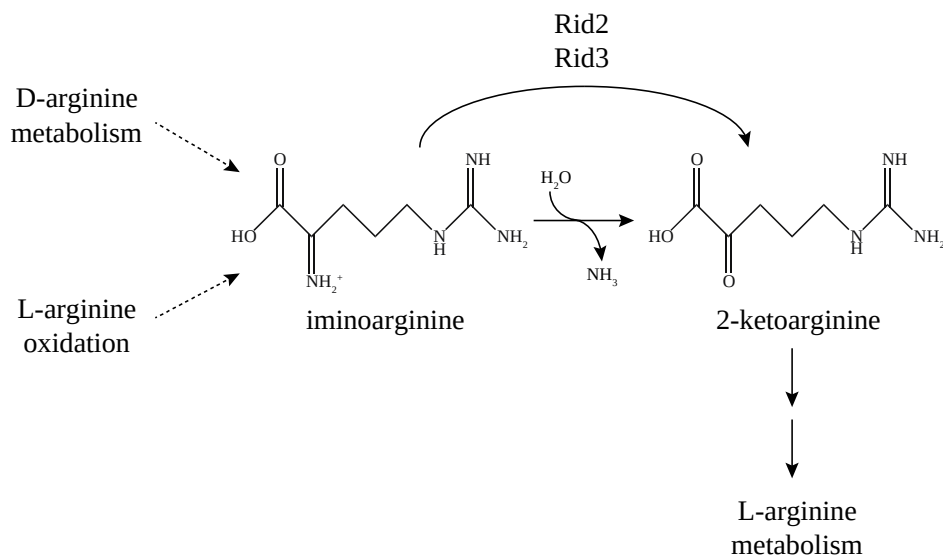


Figure 6.1 Rid2 and Rid3 iminoarginine deaminase activity. Oxidation of either arginine stereoisomer yields iminoarginine *in vitro*. Iminoarginine is deaminated via a spontaneous mechanism catalyzed by water, or is facilitated by Rid2 or Rid3 proteins. Possible *in vivo* sources of iminoarginine are indicated with dashed arrows and include D-amino acid metabolism, for which some organisms have an FAD-dependent dehydrogenase (36), or steps that catalyze L-arginine oxidation. 2-ketoarginine enters metabolism following conversion to L-arginine in *Pseudomonas aeruginosa* (36).

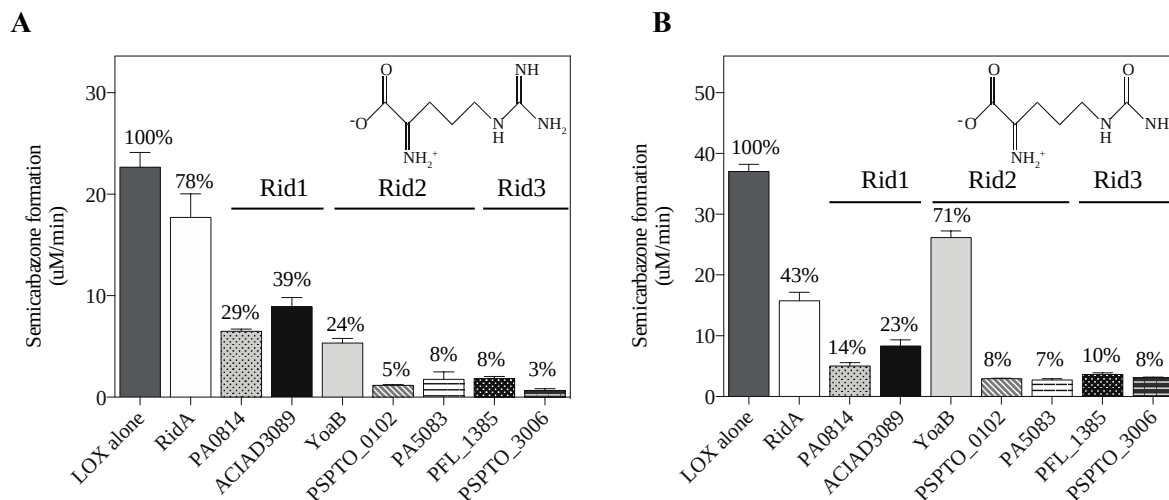


Figure 6.2 Rid deamination of LOX-derived iminoarginine and analogs. Assay mixtures (100 μ L) contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), *Crotalus adamanteus* L-amino acid oxidase (0.4 μ M), bovine liver catalase (24 Units) and the indicated Rid protein (10 μ M). Reactions were initiated with **(A)** 5 mM L-arginine or **(B)** 10 mM citrulline. The assigned Rid subfamily is indicated above the bar graph, and the predicted imine intermediate structure is shown. Reactions were monitored for five minutes at 248 nm, and the slope was used to calculate rate of semicarbazone formation over time using the molar extinction coefficient for semicarbazone ($\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$). Path lengths for each individual quartz plate well were measured immediately prior to the first reading at 248 nm. The bar graph shows the mean rate of semicarbazone formation and the error bars indicate standard deviation for three replicates. Percentages compare conditions to reactions lacking any Rid protein (LOX alone).

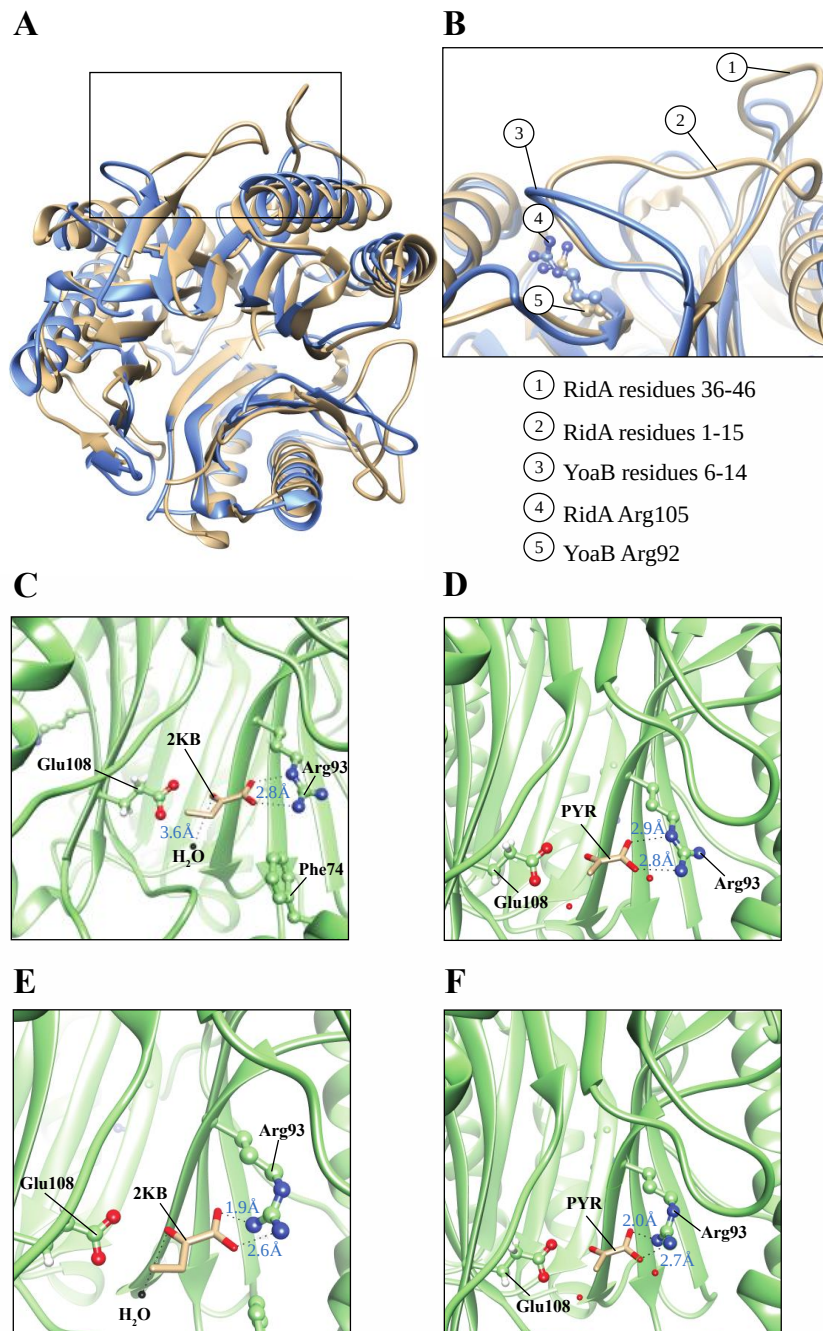


Figure 6.3 Rid protein structures

(A) The crystal structure of RidA from *E. coli* (PDB#1QU9) is shown in tan and the structure of YoaB from *S. enterica* (PDB#3GTZ) is shown in blue. Structures were aligned with the Needleman-Wunsch alignment algorithm using the UCSF Chimera package (33). (B) Features near the active site are numbered including the catalytic arginine residues, the RidA substrate-binding loop (residues 1-15), a RidA secondary loop (residues 36-46) and YoaB residues 6-14. The PA5083 structure is shown with bound 2-ketobutyrate (2KB) (C) or pyruvate (PYR) (D). The model was refined using the Dunbrack rotamer library (34) to identify Arg93 positions in closer proximity to 2-ketobutyrate (E) or pyruvate (F). Distances between the substrate and Arg93 or water were measured and are indicated in blue text.

Rid Subfamily and UniProt protein ID

Amino acid sequence

1	YP_354943	MTAA	TSQPIP-----	QGDYIPARRAGAL	MT	SGMTP	PRREGVLQFTGPVRRDQPL	EEQREAVRLA									
	ABB06492	MNRDNKLT	QIAAEFGFNPDEE---	IRIGGKYTPVLVDGNTA	MTAG	QTPRI	GDVVHFVGVVGVGSVSL	DDGRRAAGVS									
	AAY94433	MDASRKAL	FQAAAQVGHSEFE---	MRIGGNYPVPAVRNGDE	MTVSG	QTPRI	DNTVMVVGVRVGDATLE	EGIHAAEVA									
	CAG69787	MNRDDAFLT	VQAARLGYDFSGD---	MKIGGKYTSLIEHAGLA	MTSG	QTPRI	EDVKVQVCGKVGFDVSL	QAQLAASIS									
	ABB08478	MTVEHVEQK	ADLGLALPEP---	PNPLGSYTAVSEAGNL	MTVSG	QTPRI	PLVDGKVAYTGRVGEQLSV	DEGKHAARLA									
	NP_599691	MTEIAEN	RLGIELPDL---	PAPQYSYVPFN	RQGNTH	TVSG	QTPRI	DNTVMVVGVRVGDATLE	EGIHAAEVA								
	ABK85159	MQRVQTPEDK	SELEITLPAI---	RPAVGNYVSCVRVGNL	MTAG	QTPRI	QVGD-----	EYHGKLGKDIS	IEDEGYKAAQ								
	NP_249505	MQTSPAPA	IVA-----	GGAYQPVVLHAGIA	MTVSG	QTPRI	PRQHGELRWTKGVGSELDL	EQARQAARLC									
	CAL83578	MYVPVKQL	GNALMTVSG	QTPRI	PLVNGEPAY	TGKVGEERTL	EKAQKAAEIC										
	ABB09285	MANVYDK	KSLGIELPTA---	GAPAAAYVMSAQTGNT	VELSG	HHAK-	KDGKVWAGKLGADLQ	TEDGKAAARSI									
A	CAB45555	MSAVEAK	AEELGLTLPEV---	VPPLAAYQPAVQSGPY	MTVSG	QTPRI	PMLGGKLPVTGKVGA	EVTPDEAKELARTC									
	ADE01144	MSATTRCA	EELGTLTPV---	AAPVADYVPAIQTGSQ	MTVSG	QTPRI	PFVDGELTAVGKVGD	AVSAEQAAEAAARA									
	NP_599534	MASNSER	AEELGTLSPV---	AAPVADYVPAIQTGNQ	MTVSG	QTPRI	PFVDGQLPATGKVGA	EVSAEADAEKLARA									
	NP_880545	MTASISVR	AEELGLTLEPA---	NAPAAANYVPFVRDGNL	MTVSG	QTPRI	QVPRVDGKPVHLGRLGD	VSVSDEEGVQAARQA									
	AAY90595	MRDSLQQR	VOQLGLELPIP---	GOPIANYVNHAQSQNL	MTVSG	QTPRI	QAEQHPHLLGRLG	DNLSIEQGAQAAELA									
	RIDA_BACSU	MTKA	HTKHA-----	PAAIGPYSGQIIIVNNMF	MTVSG	QTPRI	PL-----	TFSGE-MVNGDIKEQ									
	RIDA_SALTY	MSKT	ATENA-----	PAAIGPYVQGVDLGSM	MTVSG	QTPRI	HPV-----	DPKTG-AVAEDVSAQ									
	NP_417583	MKKI	ETQRA-----	PGAIGPYVQGVDLGSM	MTVSG	QTPRI	HPV-----	CPQTG-EIPADVQDQ									
	NP_001267661	MAMSA	AHTLQIPASTAITRRRSITPSAGTAV	SLWRRPSPAIPS	ISSHQ	MSFKCH	AISSKCTTE	ELGIATDNA	PEALGPYSQGI	IANNLV	VVSG	SEGL	-----	IPETGQLISDD	VGEQ	-----	
	NP_005827	MSSL	LRVISTAKA---	PGAIGPYQAVLVDR	TVISG	QTPRI	IGM-----	DPSSGQLVSGGVA	E-----								
3	CAJ95231	MR	TARLLSCSSIAALATLV	VPALCIANA	AAEQV	VRHRNP	PAHMT	TEPWAREFAE	VEI	IPANAQL	TVLSG	VGP	TPVANAS	APPD	TAA	SYGDTATQ	-----
	NP_792803	MSDS	ORTSVG-----	DFPISQ	TVTVPASASL	MTVSG	QTPRI	PLADPHAPAGT	PAAYGNTQV	-----							
	AAY90670	MSLKSTV	VGGGLLFAASS	SSWAAG	ORVPSSYP	-----	NSPILQ	SVTLPANSEV	TVLSG	PLPDP	VDPKASKEQ	IIHAEGNTEA	Q-----				
	YP_002516516	MKR	LIALTVAA	CALAGAAHAQD	IVRGGDP	-----	KSPIAS	VTV	PAGYDM	TVVSG	MVPV	IDDKAV	--GVAR	FGD	TKTQ	-----	
	EEI49781	MRYK	SLVALPFV	FGVASANA	AEV	VHHKLD	-----	GMPISE	AVEINAGNNL	VELSG	KVP	TKKSADA	PEGELAS	YGNT	EEQ	-----	
	AAY47962	MFRPA	VIATAAV	VLCGGSVA	AAQAAE	TVRHKIPNS	-----	DFPIAA	AVEVPAGKA	TVVSG	KVP	AVVD	TAPKGS	AAAYG	DTNAQ	-----	
	WP_011083086	MNAND	TRIPNRNLP	-----	SKSAVT	TYHMPGGGGL	AVAVSPD	-----	RTQDI	RIQ	-----						
	NP_768102	M	KRILPY-----	EGLLHEV	VEHGGV	TVIG	GI	VAE-----	DTSLD	MSGQ	-----						
	WP_011089924	MS	TRISIR-----	TPIMHRA	VEANGF	VVFG	ST	HAD-----	DTSVS	MGDQ	-----						
	AAW34961	M	KRFDE-----	CDRMSQ	I	VF	DKYFKT	AGOVAF	-----	EP	SGDI	KKQ	-----				
2	NP_250259	M	HTRFEV-----	VKRAE	MA	LHGNT	TVHIG	SOVAD	-----	DP	SGDI	QDQ	-----				
	CAG67094	MSQSD	ORINT-----	TQVMS	SVTIFNQ	VA	MTVSG	OVPO	-----	DT	ALDI	EGQ	-----				
	WP_011086753	M	KRYVV-----	GSRMSQ	AVAAGGM	TVHIG	SOVAD	-----	DR	KAGI	VSQ	-----					
	CBJ46635	MT	ERIDP-----	EHRMSE	AVIHND	TVVYTS	-VPE	-----	NL	DEGA	EAQ	-----					
	NP_460778	MS	VRIDA-----	EDRWS	DVVIYNN	TVVYTS	-VPE	-----	NL	DADA	FEQ	-----					
	NP_416323	MT	VRIDA-----	EARWS	DVVIHNN	TVVYTS	-VPE	-----	NL	DADA	FEQ	-----					
	CAG67190	MS	ORIHV-----	EKRFE	IAISGNL	VHLAG	OLAS	-----	DT	QLDI	IKQ	-----					
	NP_253770	MEPT	RIAT-----	NDRLS	AAVCFDA	VELSG	OVPO	-----	GQ	AEDI	HGQ	-----					
	AEE68711	MS	KRFNV-----	EKRLS	DMAVYNG	VHLAG	OVPO	-----	DAS	LD	MAGQ	-----					
	ABB08209	MA	VRHHV-----	GARLSE	TAIHNGT	VHLAG	OLAS	-----	DT	TQDI	IKQ	-----					

*

Rid Subfamily and UniProt protein ID		Amino acid sequence
1	YP_354943	REAVRLACENAAARGLLAE-GESLSLHSLTVVVAEAPGFEAHSKLADFAARLREELGPA---GIGARAAGV-VATLPGNAPVEHQLIASV
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	AAY94433	RHGAQICTLRATATLEQMGD-LQRIKRIEINIVVQSAADFTQOSEVADGASAVLYSIFADA---GVHTRTSVG-VYQLPKNASVEVDIMIALHPGSGND
	CAG69787	QLAASTMTMRATATKQHYGT-LQVVEKVVIMNVVTHSTADFTQOSEVADGASLILYEILGSD--TGQHTRTSVS-VQQLPKNASVEHNFIVAILQKNNQGE
	ABB08478	KHAARLAALNVFAQRRRHSGGFG-RLRKIVRVEGHVSSADGFGQPAVIDGASDLFAAVLGDGK---AGHARSASF-QRLPADAAVIEVVAIEIDPA
	NP_599691	IHAAEVATINLFAHQSHSG--LDNVAQIVKLNIVVSSDDFIQPPQVADGASQLLEAVLGEA---GKHARTALP-TNTLPQGALVEHDAVVAVTEAAEV
	ABK85159	YKAARQSMNLNLSVVRNEFGD-LNKVKRIIVKILGEVNSTEDFIQPKVMNGASDVLVDIFGEK---GKHARSAGV-MAQLPNNTTVEHEMVLIEIE
	NP_249505	RQAARLCAACCLATEEAAGG-LQRVERIKVTVGVVSAAGFVQQPAVIDAASEYFDEVLGAR---GGHARAAGV-VAELPRGAAVEVELIAAVRP
	CAL83578	QKAAEICTINMFAAKEYFGD-LDRVVNVKILQAVNSKVGFTQQHIVVNAAQLLYDVFGD---GRHARTAVG-TNQLPLDITVEHEAVFEIETNK
	ABB09285	KAAARSIAIDLATTHAHTGD-LNKVTRIVKLMSTLNSTLDFTEQHVVINGASLILAEVFGDA---GKHARSAGF-VAQIPLGACVEHELIAEVA
	CAB45555	KELARTCALNAPAAVKSVAAGD-LDRVARIVKVVGEVSAADFTGQPGVINGASLILGEVLGDGK---GVHARSAGV-VAVLPLDAPVEVEIQVELVQP
	ADE01144	AEAAARAAALNAPAAADVAGG-LDAIKRIIVRVVEVNSAPDFTGQAGVANGASQLLGQVFGEH---GRHARSAGV-VATLPLGSAVEVELTVEV
	NP_599534	EKLARAAALNAPAAADLVG--IDKVTRIVKIVGEVSAADDFSGQPAVNGASNLMGVEVFGEA---GAHARSAGV-VAELPLNSPVEVEVVEIAQ
	NP_880545	VQAARQAALGVFAQAAATGDRLQGVARTVRLGVVFAAAPDFNRHSAIANGASDLMVNVFGEA---GRHARSAGV-VASLPNGVAVEVEAVVALAQ
	AAY90595	AQAAELAALALFAQSAAAGDDLRLQRTSRLLGVETIASADFKGHGVANGASNLLVNALGEK---GRHARTSVG-VASLPAGVAVEVDGTFELKP
A	RIDA_BACSU	-----THQVFNSTKAVTEEAGASFETVVKATVETL-----DM-EQFAEVVEVGYQYFDT---HKPARSCVE-VARLPKDALVEHEVIALVK
	RIDA_SALTY	-----ARQSFENVKALVEAAGLVGDITVKTVEVK-----DL-NDFATVATYEAFTEH-NATFPARSCVE-VARLPKDVKVEHEAIAVRR
	NP_417583	-----ARLSFENVKALVVAAGLSVGDITVKTVEVK-----DL-NDFATVATYEAFTEH-NATFPARSCVE-VARLPKDVKVEHEAIAVRR
	NP_001267661	-----TEQAQKNVGAIRAGGADYDRVKTVEVK-----NV-ADFTLVVEIKGYFPNS---PAPARSTFA-AGALPKNAKVEHDAIAVL
	NP_005827	-----AKQAQKNVGETKKAAGCDFTNVKTVEVK-----DI-NDENTVVEIKGYFPNS---NFPARAAYQ-VAALPKGSRVEHEAVIQGPLTTASL
3	CAJ95231	-----TRSVFGQMERITQDRGRYMGDITVMOALIVADPAT-GE-PDFTGFSAYKQYFGTDKQPNVPRVTRAEVLRLLVPPGWLVEHVTMAASRVPSRRPQ
	NP_792803	-----TVSVFNKTRRLTQQQLDLGLDILVLRVLRVGAETGGK-LDFAGLQAGYTOFFGTPEQLKPARTALQVVALPLPGALVEHEVAARAR
	AAY90670	-----ARVVFRKJETIASQGLTLGDVVQDLRLVVGDPALGGK-LDFDGLQVAFREFFGTQKQPLKPARTTVQVAGLVLPALVEHETVAARAR
	YP_002516516	-----TLGVFAKTEAAKSGQATMADVVMRVLVVGDPALGGK-MDFAGMMEGYRTHFGTAAQPNKPARITSQISGLVADGMLVEHEVQAAKKK
	EEI49781	-----TINVFQKTKTNENNLGLDMKDVVKMVEVLVGGREENGT-MDFKGFMEGYRTHFGTAAQPNKPARITSQISGLVADGMLVEHEVQAAKKK
2	AAY47962	-----TISVFQKTKQQLSGLGLMGDVKMVEVLVVGDPAMDGK-MDFNGFMEGYRQYFGTKEQPNLPARSAFQIAALGNPLYRVEHEVAVVRP
	WP_011083086	-----TLGAGVVDITYKDAGLDRTRIVKAEVVT-----DH-DNKPADFDEAWASWMPAG---YGPVRSFVQ--SVMPDGDLEHIIITAAALPAGTRDTSQASG
	NP_768102	-----ANDVFGQSRSLKTLGSDLANVTVTTEVT-----NL-GEKAAFNAAKAHFAEA---HLPARAVVG-VADLGGGVKVEHTALAAAPTSS
	NP_011089924	-----TRNIDGKTAGYKKEAGTDKSRVVSASAEV-----DL-SKKKEMDAAWTEFFGD---NLPTRATVG-VADLGGSALEHVVVIALKG
	AAW34961	-----TKEADAEFDALFEKIGASKGDLTQIQVLA-----NM-QDFDAMJETYDAWIKNY---PKPVRAVVG--SALAEGYLVEHQAFGKLEN
	NP_250259	-----TRQILENIDRLTQSVGSDRGQVSVRLA-----HR-EDYAGLQVVDQWFFPEG---RAPTRACSL-AELIDPRWRVEHIVVAARQ
	CAG67094	-----TRDVSTIDQLTAAANSDKSRILSVQVPLK-----NL-SDFPKVVAIWMWELDGL---ATPARATIQ-ADLVNPDWLVEHIAVIAAQ
	WP_011086753	-----TQQVFAKIDALKEAGTDKRSKILAVNVPL-----HI-TDFDTMSVYDSWIDPA---NPPARACVE-ARLADPDLRVEHTAVALL
	CBJ46635	-----TANADATDKLITRVGSDKSKILDATTEV-----HK-EDFAAMIRAWDAWVSPG---HAPVRCVQ-ADLMDEKYRVEHKIIAAI
	NP_460778	-----TANTFAQIDAVEKQGSKSKRIIDATTEV-----DK-ADFAAMIKAWDAWVAG---HAPVRCVQ-AGLMNPKYKVEHKIVAAV
	NP_416323	-----TANTFAQIDAVEKQGSNKSSTIDATTEV-----DK-NDFAAMIKAWDAWVAG---HAPVRCVQ-AGLMNPKYKVEHKIVAAV
	CAG67190	-----TQQTEDIDRFDADAGTDKSHHVSVMPLK-----DIEHDYDAMIEIWDACADI---QALPRTCTVE-AKMYRPDVLVEHTVAVKP
	NP_253770	-----TREVFQKIDALFAEAGSRKERITTSATTEV-----DIARDFAALVEVWTQWLP TG---QAPSRRTVQ-AELARPSVLVEHTVVAARG
	AEF68711	-----TAQVFATIDRLFAEAGTDKTKIIMAQIIVA-----NM-KEFDGMIKAWDAWVAPG---NSPPRATVE-ARLANPDYKVEHIVVTAAG
	ABB08209	-----TREVGHIIDRLFAEANSDKAHILSVQVLAIS-----DL-ANFEGMIAEWDAWVAHG---NTPPRATVE-AKLADPALLVEHVVVAQRS
	NP_715977	-----TTTMTSEEVDALFAQAGSSREHHSATTEV-----DM-NDYDAMIAVWDWVVPKG---HAPARACVQ-AAIAPEYLVEHVSIAAVAKA
	ABB10180	-----ATEIITRIDALFAAGVVDKTRVITANVLS-----DA-AHFDAFIAVWDWVVPAG---HAPTRACVQ-ALLMKPGLDVEHIAVIALA
	NP_789961	-----TREVNSTIERLIDLAGTDKTRITTSVTVLK-----DIDADFEGMIRVWDQWLPKG---FAPARATVE-AKLCEPQILVEHISVIAALP
	NP_253990	-----TRETFAAIERLIEEAGTDKTRITTSVTVLK-----DIDADFEGMIRVWDQWLPKG---VAPARATVE-AKLCEPEILVEHISVIAALP
	NP_767767	-----TQDIEATIDGHFAKAGTDKSKILSATTEV-----DM-KTFEGMIAVWDWVSPG---NTPPARATVE-AKLAAPQYTVEHMTAAK
	YP_352792	-----TRTCFAEVDRLTALAGTDKTRITTSVTVLK-----DM-ADFAEMIAVWDWVVPQ---NCPARATGE-AKLAAPDYRVEHIVVAVL
	AAV96190	-----TRDCFAEVDALFAEAGSDNTRITTSVTVLK-----DM-ADFAEMIAVWDWVVPAG---HAPARACGE-AKLARDTLKVEHIVVTAACD
	AAV95275	-----TQDCFNQIDALFSEAGSDRTRITTSVTVLK-----DM-ADFAEMIAVWDWVVPAG---HAPARACGE-ARLATPDYRVEHIVVTAARAD

§

Figure 6.4. Amino acid alignment. An amino acid alignment of Rid proteins from subfamilies RidA, and Rid1-3 shows the residues analogous to RidA Tyr-17. Amino acid sequences were accessed and aligned using Geneious software and MUSCLE alignment settings. Proteins were assigned to subfamilies using the PubSEED database (39) based on the subsystem originally published by Niehaus, *et al.*, 2015 (15). Similar residues are boxed in grey and identical residues are boxed in black. Residues analogous to RidA are indicated as follows: Tyr17 (*) and Arg105 (§).

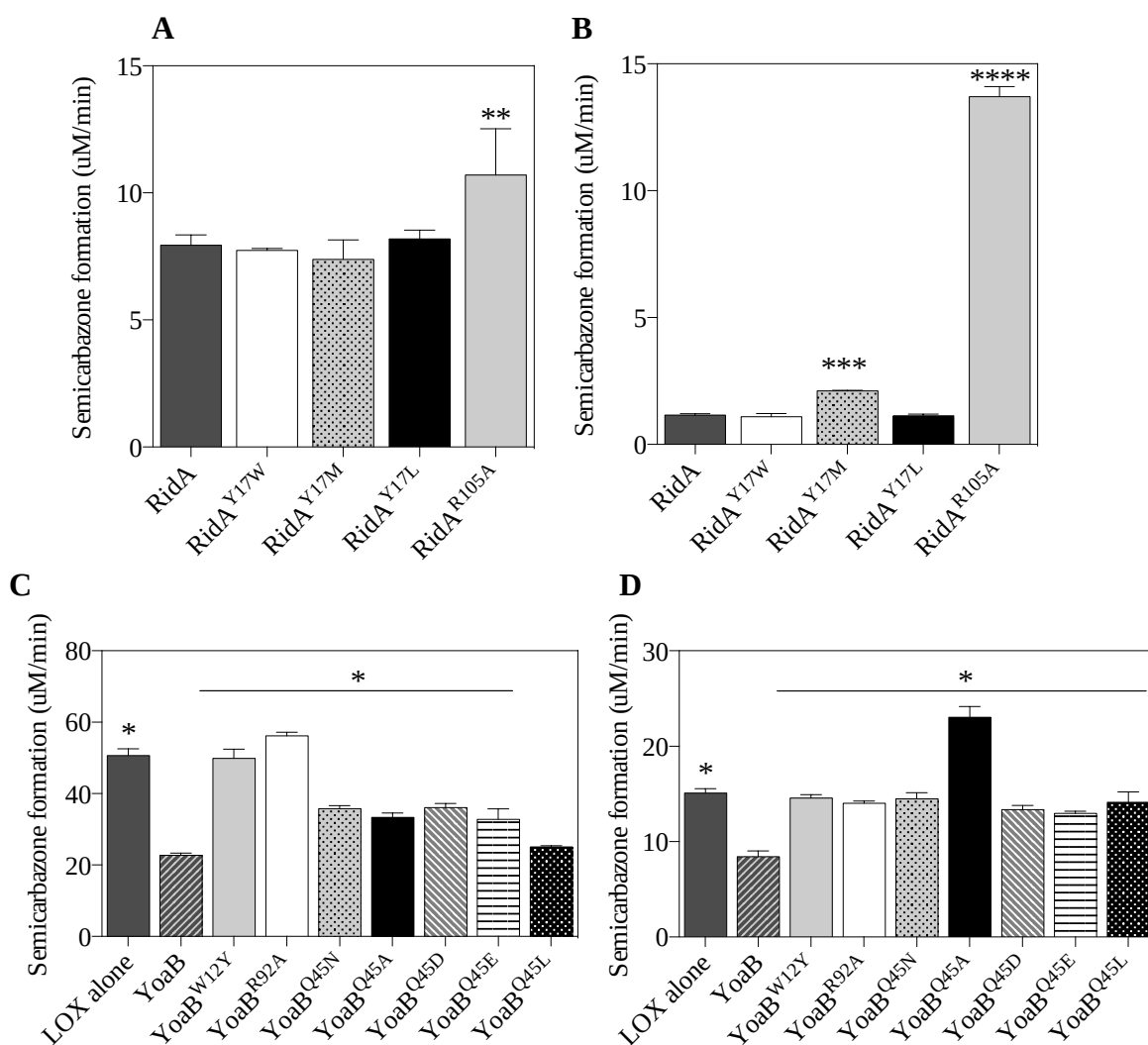


Figure 6.5 Rid protein variant activity

Assay mixtures (100 μ L) contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), bovine liver catalase (24 Units), the indicated Rid protein (10 μ M) and either *Crotalus adamanteus* L-amino acid oxidase (0.4 μ M, Panels A-C) or D-amino acid oxidase from porcine kidney (10 milli-Units, Panel D). RidA reactions were initiated with (A) 5 mM L-arginine or (B) 10 mM 2-aminobutyrate. YoaB reactions were initiated with (C) 5 mM L-arginine or (D) 10 mM D-alanine. Reactions were monitored for five minutes at 248 nm, and the slope was used along with the measured path length to calculate rate of semicarbazone formation over time using the molar extinction coefficient for semicarbazone ($\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$). The bar graph shows the mean rate of semicarbazone formation and the error bars indicate standard deviation for three replicates. An ordinary one-way ANOVA statistical analysis was performed to compare activity for each condition to wild type protein (RidA or YoaB) and an asterisk (*) indicates $p < 0.05$.

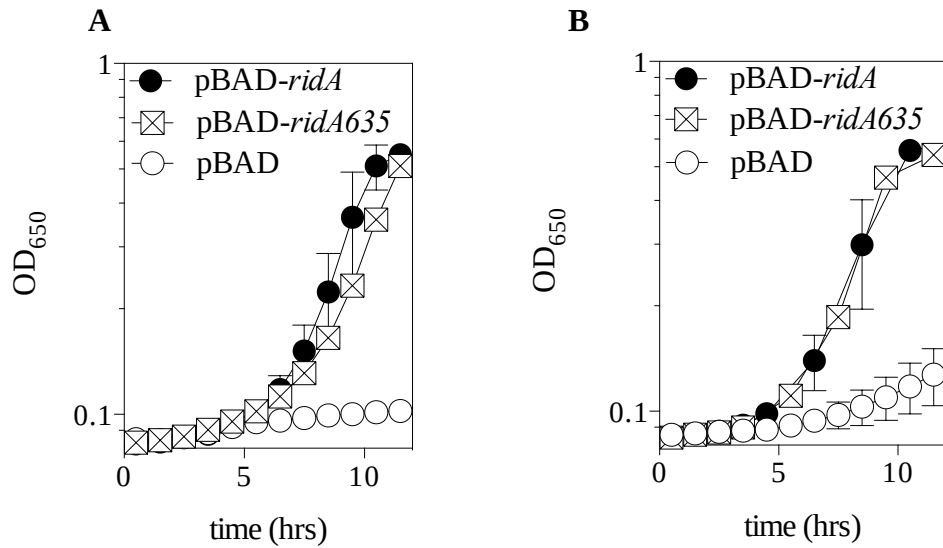


Figure 6.6 *RidA*^{ΔSer2-Gly15} variant functions *in vivo*.

A *S. enterica* *ridA* strain was transformed with pDM1439 (pBAD24-*ridA*), pDM1471 (pBAD24-*ridA635*, encoding *RidA*^{ΔSer2-Gly15}), or an empty vector. Growth was assessed in minimal medium containing glycerol and 0.2% arabinose with **(A)** 5 mM serine or **(B)** 250 uM cysteine.

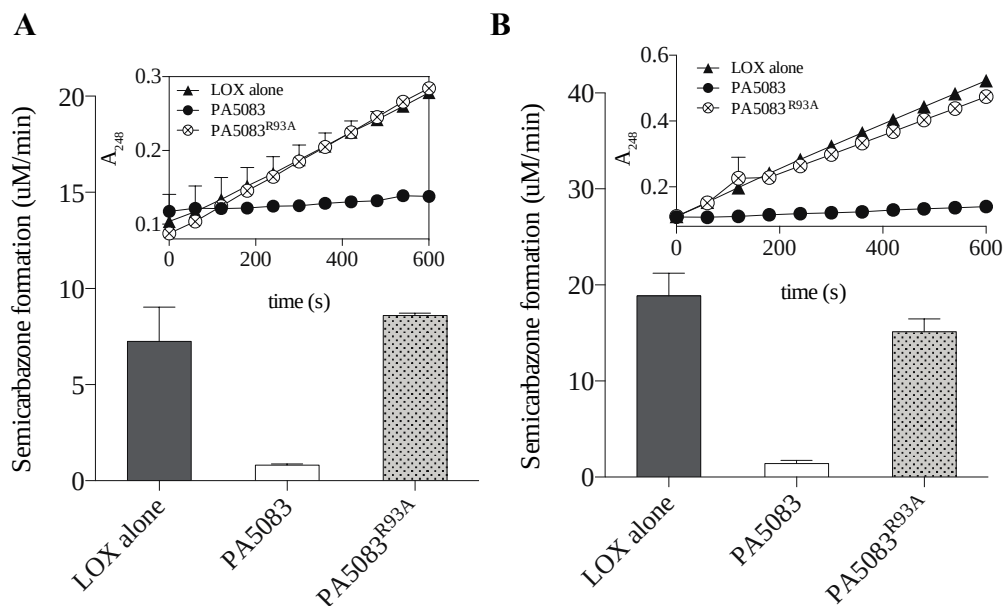


Figure 6.7 PA5083 variant activity. Wild type PA5083 and variant PA5083^{R93A} activity was tested using the LOX assay to generate **(A)** iminoarginine or **(B)** iminobutyrate. The bar graph shows the rate of semicarbazone formation, which was calculated from the rate of product formation at 248 nm depicted in the inset above the bar graph.

Table 6.1 Strains and plasmids

Strain	Relevant genotype ^a	Encodes	Derivative of
<i>S. enterica</i> strains ^b			
DM12920	<i>ridA1::Tn10d</i> (Tc)		
DM14846	<i>ridA1::Tn10d</i> (Tc)	pBAD24 empty vector	pBAD24
DM14847	<i>ridA1::Tn10d</i> (Tc) pDM1439	<i>S. enterica</i> RidA	pBAD24
DM15408	<i>ridA1::Tn10d</i> (Tc) pDM1386	<i>S. enterica</i> YoaB	pBAD24
DM15441	<i>ridA1::Tn10d</i> (Tc) pDM1471	<i>S. enterica</i> RidA ^{ΔSer2-Gly15}	pBAD24
<i>E. coli</i> strains ^c			
DM12740	pET20b- <i>ridA</i>	<i>S. enterica</i> RidA	pET20b
DM13042	pET20b- <i>ridA652</i>	<i>S. enterica</i> RidA ^{R105A}	pET20b
DM14338	pDM1387	<i>S. enterica</i> YoaB	pET20b
DM14674	pDM1416 (pET20b- <i>yoaB627</i>)	<i>S. enterica</i> YoaB ^{W12Y}	pET20b
DM14675	pDM1417 (pET20b- <i>yoaB628</i>)	<i>S. enterica</i> YoaB ^{Q45N}	pET20b
DM14740	pDM1423 (pET20b- <i>yoaB30</i>)	<i>S. enterica</i> YoaB ^{R92A}	pET20b
DM14741	pDM1424 (pET20b- <i>yoaB631</i>)	<i>S. enterica</i> YoaB ^{Q45A}	pET20b
DM14742	pDM1425 (pET20b- <i>yoaB632</i>)	<i>S. enterica</i> YoaB ^{Q45E}	pET20b
DM14743	pDM1426 (pET20b- <i>yoaB633</i>)	<i>S. enterica</i> YoaB ^{Q45D}	pET20b
DM14744	pDM1427 (pET20b- <i>yoaB634</i>)	<i>S. enterica</i> YoaB ^{Q45L}	pET20b
DM15038	pDM1446 (pET20b- <i>ridA635</i>)	<i>S. enterica</i> RidA ^{ΔSer2-Gly15}	pET20b
DM15324	pDM1459	<i>P. aeruginosa</i> PA0814	pET28
DM15325	pDM1460	<i>P. fluorescens</i> PFL_1385	pET28
DM15488	pDM1478	<i>P. syringae</i> PSPTO_3006	pET28
DM15489	pDM1477	<i>P. syringae</i> PSPTO_0102	pET28
DM15564	pDM1488	<i>A. baylyi</i> ACIAD3089	pET28
DM15686	pDM1514	<i>P. aeruginosa</i> PA5083	pET28
DM16063	pDM1547 (pET28- <i>ridA653</i>)	<i>S. enterica</i> RidA ^{Y17W}	pET20b
DM16064	pDM1548 (pET28- <i>ridA654</i>)	<i>S. enterica</i> RidA ^{Y17M}	pET20b
DM16065	pDM1549 (pET28- <i>ridA655</i>)	<i>S. enterica</i> RidA ^{Y17L}	pET20b
DM16066	pDM1550	<i>P. aeruginosa</i> PA5083 ^{R93A}	pET28
Plasmid	Description or relative genotype		
pBAD24	P _{araBAD} expression vector, <i>bla</i> ⁺ (40)		
pBAD24 (pCV1)	P _{araBAD} expression vector, <i>bla</i> ⁺ modified for BspQI cloning (28)		
pET20b	T7 promoter, C-terminal His ₆ overexpression vector, <i>bla</i> ⁺ (Novagen)		
pET28	T7 promoter, C-terminal His ₆ overexpression vector, <i>kan</i> ⁺ (27)		

^a Tn10d(Tc) refers to the transposition-defective mini-Tn10 (Tn10Δ16Δ17) (41)

^b *Salmonella enterica* strains are derivatives of *Salmonella Typhimurium* serovar LT2

^c *Escherichia coli* strains are derivatives of BL21AI and were used to purify relevant proteins

Table 6.2 Primers

Primer name/relevant plasmid	Primer sequence (5' to 3')
PR219 (pDM1386)	AGAGGAATTCATGTCTATCGTGC
PR220 (pDM1386)	TCTCAAGCTTTTATAACCGCCG NNGCTCTTCNTTCATGCCATACGTACAGGGCGTTG
PR599 (pDM1471, pDM1446)	A
PR600 (pDM1471, pDM1446)	NNGCTCTTCNTTATTAGCGACGAACAGCGATC ATCACCACGTCTGAATAACGATCTTCGGCATCAAT
PR257 (pDM1416)	ACGCAC GTGCGTATTGATGCCGAAGATCGTTATTCAGACGT
PR258 (pDM1416)	GGTGAT
PR259 (pDM1417)	CCAGCACCGCATCAATATTCGCCAGAGTATTCGCC
PR260 (pDM1417)	GGCGAATACTCTGGCGAATATTGATGCGGTGCTGG
PR269 (pDM1423)	CCATGCGCCGGTAGCCTGTACCGTACAGG
PR270 (pDM1423)	CCTGTACGGTACAGGCTACCGGCGCATGG
PR271 (pDM1424)	GAATACTCTGGCGGCAATTGATGCGGTGC
PR272 (pDM1424)	GCACCGCATCAATTGCCGCCAGAGTATTC
PR273 (pDM1425)	GAATACTCTGGCGGAAATTGATGCGGTGC
PR274 (pDM1425)	GCACCGCATCAATTTCCGCCAGAGTATTC
PR275 (pDM1426)	GAATACTCTGGCGGACATTGATGCGGTGC
PR276 (pDM1426)	GCACCGCATCAATGTCCGCCAGAGTATTC
PR277 (pDM1427)	GAATACTCTGGCGCTAATTGATGCGGTGC
PR278 (pDM1427)	GCACCGCATCAATTAGCGCCAGAGTATTC
PR961 (pDM1547)	GTCAACGCCCTGTACCCATGGGCCGATTGCTG
PR962 (pDM1548)	GGTCAACGCCCTGTACCATTTGGGCCGATTGCTGCT
PR963 (pDM1549)	GGTCAACGCCCTGTACTAATGGGCCGATTGCTGC
PR964 (pDM1550)	CTGGACGGTGGTTCGCGCTGGGTGCCTGG
YjgF 105A For (pET20b- <i>ridA652</i>)	CAACGCCACCTTCCCGGCGGCTTCCTGCGTGGAAG GACGGGCAACTTCCACGCAGGAAGCCGCCGGGAA
YjgF 105A Rev (pET20b- <i>ridA652</i>)	GG
YoaB For <i>NdeI</i> (pDM1387)	GAGACATATGTCTATCGTGCGTATTG
YoaB Rev <i>XhoI</i> (pDM1387)	GAGACTCGAGTACCGCCGCGACAATC

Table 6.3 YoaB iminoarginine deaminase activity^a

Rid protein	Rate of semicarbazone formation ^b		
None	18 ± 1.1	35 ± 1.1	41 ± 1.1
YoaB	4 ± 0.3	11 ± 0.3	19 ± 1.3
Ratio ^c	22%	32%	46%
	2 mM	5 mM	10 mM
	L-arginine		

^a Iminoarginine was generated via *Crotalus adamanteus* L-amino acid oxidase (40 uM) in an assay mixture (100 µL) that contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), bovine liver catalase (24 Units). When indicated, YoaB was present at 10 µM. Reactions were initiated with the indicated concentration of L-arginine and product formation was measured at 248 nm. The slope was used to calculate the rate of semicarbazone formation over time using the molar extinction coefficient for semicarbazone ($\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$). Path lengths for each individual quartz plate well were measured immediately prior to the first reading at 248 nm.

^b Values indicate the average rate ± standard deviation from three replicates

^c The ratio of the rates obtained from the two conditions (YoaB over no Rid) is expressed as a percentage

Table 6.4 Truncation of RidA substrate binding loop affects *in vitro* activity^a

Rid protein	Rate of semicarbazone formation ^b						
None	52 ± 2.0	46 ± 0.7	18 ± 0.6	21 ± 0.9	32 ± 0.3	18 ± 1.1	22 ± 2.3
RidA	3 ± 0.1	6 ± 0.4	3 ± 0.2	16 ± 1.3	4 ± 0.3	12 ± 0.3	19 ± 0.1
RidA ^{ΔSer2-Gly15}	48 ± 3.7	46 ± 1.8	14 ± 0.6	21 ± 1.6	29 ± 0.5	15 ± 0.4	21 ± 0.4
	2AB	Leu	Gln	Phe	Met	Arg	His
	LOX substrate						

^a The assay mixture (100 μL) contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), bovine liver catalase (24 Units), *Crotalus adamanteus* L-amino acid oxidase (40 uM) and the indicated RidA protein (10 μM). Reactions were initiated with L-amino acid (10 mM), 2-aminobutyrate (10 mM) or L-arginine (2 mM) and product formation was measured at 248 nm. The slope was used to calculate the rate of semicarbazone formation over time using the molar extinction coefficient for semicarbazone ($\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$). Path lengths for each individual quartz plate well were measured immediately prior to the first reading at 248 nm.

^b Values indicate the average rate ± standard deviation from three replicates

CHAPTER 7

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

7.1 SUMMARY AND CONCLUSIONS

The goal of this work was to explore the breadth of the ubiquitous Rid (YjgF/YER057c/UK114) protein superfamily. Bioinformatics and phylogenetic analyses divided the family into eight subgroups (RidA and Rid1-7). Prior to this work, the sequence-diverse members of the superfamily were united until the establishment of subfamilies highlighted the disparity in our understanding of the Rid1-7 subfamilies, which are found exclusively in prokaryotes (1). The archetypal protein RidA from *Salmonella enterica* is a 2-aminoacrylate deaminase that preempts metabolic stress, which is the defining activity for the RidA subfamily. The Rid4-7 proteins lack the critical active site residue and are therefore not expected to catalyze a deaminase reaction. This prediction was supported by the observation that purified STM1549 (Rid7) from *Salmonella enterica* lacked activity *in vitro* (1). Our understanding of the Rid1-3 subfamilies comes primarily from biochemical data (1, 2) and recently solved crystal structures.

Deaminase activity is conserved in Rid1-3 subfamilies

The work in Chapter 3 demonstrated that deaminase activity is broadly conserved in Rid1, 2, and 3 subfamilies, which supported the idea that the active site arginine was necessary and sufficient for activity. A subset of the tested proteins functioned *in vivo* despite universal 2AA deamination *in vitro*. This discrepancy differentiated RidA from the other Rid subfamilies. Combined with previous observations that all tested eukaryotic, archaeal, and bacterial RidA proteins successfully substituted for *S. enterica* RidA *in vivo*, the broad Rid substrate range *in*

vitro was an indication that non-RidA proteins might deaminate a non-2AA metabolite *in vivo*. Taken together, these findings supported the idea that particular RidA proteins act as “housekeeping” deaminases that primarily respond to 2AA stress, while Rid1-3 proteins might be specialized deaminases.

FAD-dependent enzymes generate Rid substrates

Genomic synteny suggested a link between FAD-dependent enzymes and Rid proteins, which was supported by biochemical evidence. D-arginine dehydrogenase DauA from *Pseudomonas aeruginosa* was identified as the third mechanism to generate substrates for Rid2 and Rid3 enzymes (2). Prior to this, candidate enamine/imine generators in bacteria were exclusively PLP-dependent enzymes that released 2AA. FAD-dependent eukaryotic amino acid oxidases generated imine Rid substrates *in vitro* (1), and the DauA-generation of iminoarginine suggested that FAD-dependent imine production might be relevant in bacteria. There is no evidence to suggest that metabolic stress accompanies the production of other inherently reactive enamines/imines. However, it is likely that 2AA is not unique in its ability to influence the metabolic network. FAD-dependent imine generators are likely distinct from the PLP-dependent sources of 2AA, and the physiological relevance of many Rid imine substrates has not been explored. Any mutant phenotypes associated with production of non-2AA Rid substrates are expected to be subtle, and elucidation of the physiological substrates for Rid1, Rid2, and Rid3 subfamilies might require inactivation of multiple genes due to the potential for compensatory activities across Rid subfamilies.

Metabolic networks can be configured to indirectly quench 2AA

In Chapter 4, a putative aspartate/glutamate racemase (MMP0739) from a methanogenic archaeon was identified that substituted for *S. enterica* RidA. In addition, a homologous protein

(YgeA) from *Escherichia coli* and *S. enterica* similarly restored metabolic balance in a *ridA* mutant strain. The identification of a compensatory activity from *S. enterica* was surprising, and suggested that future screens for multi-copy suppressors with inducible promoters might uncover additional 2AA quenching mechanisms such as YgeA. 2AA is an inherently reactive molecule that is likely to interact with metabolites in addition to the enzymes it inactivates. YgeA and MMP0739 lacked the canonical cysteinyl residues that catalyze PLP-independent racemization, and the data suggested these proteins belong in a separate class. Together these observations were consistent with a model where MMP0739 and YgeA produce a carbanion intermediate that quenches 2AA. As *Methanococcus maripaludis* lacks any encoded Rid proteins, if the organism does experience 2AA stress, a metabolite-2AA quenching mechanism is likely.

***Pseudomonas aeruginosa* experiences 2-aminoacrylate stress**

Chapter 5 describes the mutant phenotype of a *Pseudomonas aeruginosa* strain lacking RidA homolog PA5339. Complementation studies indicated the mutation in PA5339 was causative, and purified PA5339 demonstrated 2AA deaminase activity *in vitro*. The observation that a phenotype arises in the absence of a single mutation was surprising, given that *P. aeruginosa* genome encodes multiple RidA homologs, including PA0814, PA5083, and PA3123, which each restored metabolic balance in a *S. enterica ridA* mutant strain. Along with nutritional analyses, the data suggested the metabolic networks of a *P. aeruginosa* Δ PA5339 mutant differ from *S. enterica ridA* strains.

The active site arginine is required for iminoarginine deaminase activity

In Chapter 6, I showed that the highly conserved active site arginine residue was necessary and sufficient for iminoarginine deaminase activity. The crystal structure of Rid2 protein PA5083 from *Pseudomonas aeruginosa* provided insights into the interaction between

Arg93 and bound substrate. Ketoacid soaking experiments suggested that the rotameric conformation of either the Arg93 side chain or the substrate itself might influence iminoarginine binding. Taken together, these studies showed the deaminase mechanism is conserved within the Rid family acting on diverse substrates. Additionally, I propose that ketoacids be routinely screened in ligand studies of the Rid family, due to their general stability (relative to enamine/imine species) and the ability to bind relevant the active site residue.

A comprehensive Rid model

The data support a model where Rid proteins facilitate intermediate deamination that would otherwise occur slowly in a spontaneous reaction catalyzed by solvent water (Figure 7.1). This model encompasses the role of RidA as a 2AA stress response protein where 2AA quenching by water alone is not sufficient to spare damage to cellular enzymes. In this model, various PLP-dependent (3-5) or FAD-dependent enzymes (2) might give rise to reactive intermediates that are hydrolyzed by water at a slow rate. Rid activity accelerates the rate of this deamination step, facilitating the pathway. This is consistent with described PLP- and FAD-enzyme mechanisms, in which an intermediate is released and reaction with water yields the final product (6). Historically, *in vivo* water was assumed to be present in excess. However, the cell likely has low water availability due to crowding and possible water sequestration (3, 7, 8). Previous biochemical characterization of PLP-enzymes (3-5) and the data presented here suggest PLP- and FAD-derived intermediates might persist longer *in vivo* than *in vitro*.

7.2 FUTURE DIRECTIONS

Explore the connections between FAD-enzymes and the Rid paradigm

FAD-dependent generation of Rid substrates *in vitro*, along with gene synteny, suggests a functional connection *in vivo*. In addition to DauA, *P. aeruginosa* encodes FAD-dependent

oxidase DguA (D-glutamate dehydrogenase EC 1.4.5.1), in the D-glutamate utilization operon along with DguB (PA5083, Rid2). I hypothesize DguA will release iminoglutarate from D-glutamate and predict that DguB will accelerate the formation of 2-ketoglutarate, based on the conservation of imine formation in the FAD-enzyme mechanism and the imine deaminase activity of DguB *in vitro* (2). It is possible that subtle growth defects might arise in a *dguB* mutant strain due to compromised D-amino acid metabolism. The *P. aeruginosa* genome includes multiple putative imine generators (i.e. dehydratases, dehydrogenases, or oxidases) in proximity to encoded Rid proteins that might suggest pathways that rely on Rid proteins for ketoacid formation. PLP-dependent enzymes exclusively generate 2AA stress, and there is no indication that FAD-derived imines will be detrimental to growth. Instead, as imines accumulate, the cell might become limited for the corresponding ketoacid, which will likely lead to subtle growth defects in the absence of Rid proteins.

Probe the Rid deaminase mechanism

An arginine residue was required for Rid deaminase activity (3), which was consistent with a mechanism in which the arginine guanidinium group engages in hydrogen bonding with substrate carboxyl group. This interaction suggested that Rid protein residues contribute to binding but not catalysis, and water molecules in the active site catalyze deamination. It was not possible to determine the tautomer species (i.e. enamine or imine) of the substrate from the biochemical or structural data. Capturing this catalysis is complicated by the short half-life of enamines/imines. The biochemical data indicated that enzymes that generate either the enamine (PLP-dependent) or imine (FAD-dependent) produce Rid substrates, so it is possible that either species binds within the Rid active site. Enamine to imine tautomerization involves addition of a proton and is a necessary step in the ultimate conversion to ketoacid. If the enamine species

binds to the active site, then a proton must be donated from either the enzyme or a water molecule, and a second water molecule would be required for subsequent deamination. The initial proton would not be required if tautomerization occurs in solution. Establishing comparative measures for Rid protein activity (i.e. enzyme kinetics) and testing the rate of product release will help to refine the mechanism.

Investigate the role of extracellular RidA

RidA proteins have been implicated in cancer biology (9) and allergies to the house dust mite (10) suggest the proteins may exist outside the cell. It is also possible that when a bacterial cell or a dust mite (*Dermatophagoides farinae*) is lysed, RidA (Der f 34) is released into the host where antibodies against the protein can develop. In either scenario, it will be important to determine whether RidA is active and if 2AA deamination, or 2AA stress, is implicated in these processes. RidA proteins appear to be physically sturdy, as the pioneer purification schemes were based on the perchloric acid solubility and purified bacterial Rid proteins retain activity following freeze-thaw cycles, which raises questions about whether a RidA protein might retain activity following lysis of a bacterial cell within a host. Even if RidA proteins are denatured or inactive, the presence of dust mite RidA proteins has been implicated in precipitating an allergic response in humans and it is possible that foreign RidA proteins could serve as a signal to hosts.

Probe Rid redundancy in *Pseudomonas aeruginosa*

Of the nine annotated *P. aeruginosa* Rid proteins, four functional 2AA deaminases (PA0814, PA5083, PA5339 and PA3123) have been described (2). As this activity has been conserved in all RidA proteins tested, the expectation is that RidA proteins PA3499 and PA5392 will catalyze this reaction *in vitro*. The mutant phenotypes of a strain lacking PA5339 suggest that the additional Rid proteins serve a separate functional role. Genetic experiments along with

the biochemical activities and putative operon structures suggest potential pathways that might involve Rid proteins. Understanding the apparent redundancy in *P. aeruginosa* will likely uncover the functional roles of Rid1-3 proteins and elucidate the differences among *P. aeruginosa* RidA proteins and the differences between *P. aeruginosa* and *S. enterica*.

Consider cellular chemistry in a low-water environment

Elucidation of RidA suggested water is insufficient to catalyze certain reactions *in vivo* (Jeff Flynn, thesis; Lambrecht 2012; Downs, 2015; Kreuzer-Martin, 2006; Zhou, 2008]. The effect of RidA is apparent in multiple *in vitro* scenarios, where water is present in excess. *In vivo*, this was demonstrated by the deleterious consequences of 2AA accumulation, which arises in the absence of a functional RidA in multiple organisms (9, 11-15). In a second *in vivo* scenario that did not elicit metabolic stress, accumulation of the reactive metabolite 2-aminocrotonate (2AC) benefitted the metabolic network by promoting formation of the thiamine precursor phosphoribosylamine (PRA) (16). Both of these situations were uncovered by a mutation in *ridA* that limited conversion of the enamine to the ketoacid, and showed that water was slow to catalyze this reaction. It is likely that other metabolic nodes benefits from facilitated ketoacid formation catalyzed by Rid proteins. These pathways may involve imine production via PLP- or FAD-dependent mechanisms.

Work in Chapter 4 and done by others (Ernst and Christopherson, unpublished) suggests networks can be configured to quench reactive intermediates through metabolite-metabolite interactions, and these contributions are assumed to be small *in vivo* because of expectations set *in vitro*. These types of reactions are consistent with the metabolic robustness that was uncovered by studying *S. enterica* RidA and are likely more common than currently realized. The activities of YgeA and MMP0739 suggested the carbanion intermediate might be released from the

enzyme active site. The ideas presented here are consistent with a robust metabolic network. In contrast to what is predicted from *in vitro* studies, catalytic intermediates may be released *in vivo*, and it is likely that these nuances contribute to overall integration of the metabolic network.

7.3 REFERENCES

1. Niehaus TD, Gerdes S, Hodge-Hanson K, Zhukov A, Cooper AJ, ElBadawi-Sidhu M, et al. Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC genomics*. 2015;16:382.
2. Hodge-Hanson KM, Downs DM. 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*. 2017;12(9):e0185544.
3. Lambrecht JA, Flynn JM, Downs DM. Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions. *The Journal of biological chemistry*. 2012;287(5):3454-61.
4. Ernst DC, Lambrecht JA, Schomer RA, Downs DM. Endogenous synthesis of 2-aminoacrylate contributes to cysteine sensitivity in *Salmonella enterica*. *Journal of bacteriology*. 2014;196(18):3335-42.
5. Ernst DC, Anderson ME, Downs DM. L-2,3-diaminopropionate generates diverse metabolic stresses in *Salmonella enterica*. *Molecular microbiology*. 2016;101(2):210-23.
6. Eliot AC, Kirsch JF. Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. *Annual review of biochemistry*. 2004;73:383-415.
7. Kreuzer-Martin HW, Lott MJ, Ehleringer JR, Hegg EL. Metabolic processes account for the majority of the intracellular water in log-phase *Escherichia coli* cells as revealed by hydrogen isotopes. *Biochemistry*. 2006;45(45):13622-30.
8. Zhou HX, Rivas G, Minton AP. Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu Rev Biophys*. 2008;37:375-97.
9. Downs DM, Ernst DC. From microbiology to cancer biology: the Rid protein family prevents cellular damage caused by endogenously generated reactive nitrogen species. *Molecular microbiology*. 2015;96(2):211-9.
10. ElRamlawy KG, Fujimura T, Baba K, Kim JW, Kawamoto C, Isobe T, et al. Der f 34, a Novel Major House Dust Mite Allergen Belonging to a Highly Conserved Rid/YjgF/YER057c/UK114 Family of Imine Deaminases. *The Journal of biological chemistry*. 2016;291(41):21607-15.

11. Enos-Berlage JL, Langendorf MJ, Downs DM. Complex metabolic phenotypes caused by a mutation in *yjgF*, encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of bacteriology*. 1998;180(24):6519-28.
12. Leitner-Dagan Y, Ovadis M, Zuker A, Shklarman E, Ohad I, Tzfira T, et al. CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. *Planta*. 2006;225(1):89-102.
13. Niehaus TD, Nguyen TN, Gidda SK, ElBadawi-Sidhu M, Lambrecht JA, McCarty DR, et al. Arabidopsis and maize RidA proteins preempt reactive enamine/imine damage to branched-chain amino acid biosynthesis in plastids. *Plant Cell*. 2014;26(7):3010-22.
14. Kim JM, Yoshikawa H, Shirahige K. A member of the YER057c/yjgF/Uk114 family links isoleucine biosynthesis and intact mitochondria maintenance in *Saccharomyces cerevisiae*. *Genes to cells : devoted to molecular & cellular mechanisms*. 2001;6(6):507-17.
15. Oxelmark E, Marchini A, Malanchi I, Magherini F, Jaquet L, Hajibagheri MA, et al. Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome. *Molecular and cellular biology*. 2000;20(20):7784-97.
16. Lambrecht JA, Downs DM. Anthranilate phosphoribosyl transferase (TrpD) generates phosphoribosylamine for thiamine synthesis from enamines and phosphoribosyl pyrophosphate. *ACS Chem Biol*. 2013;8(1):242-8.

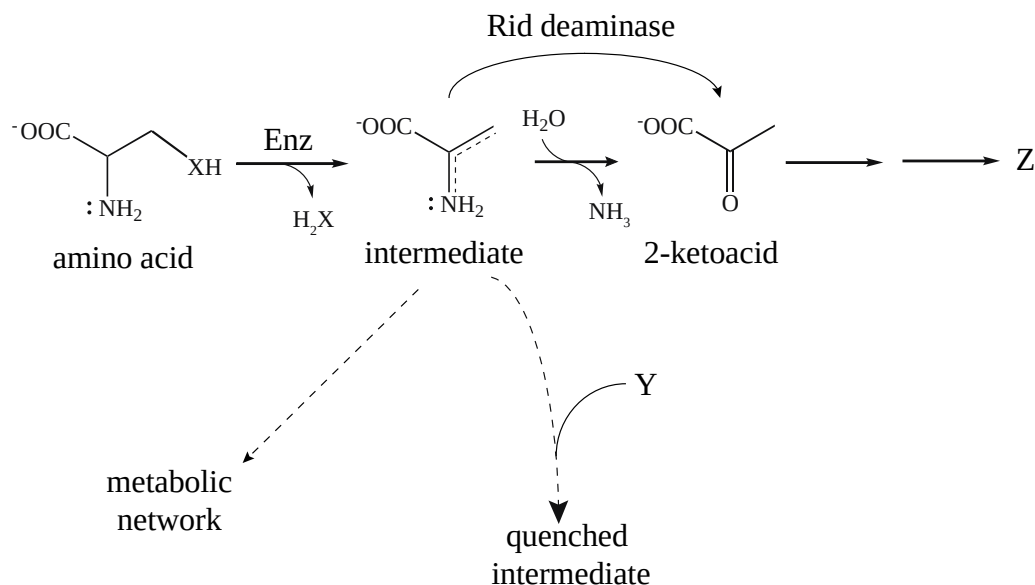


Figure 7.1 Model for Rid deaminase activity in facilitated ketoacid formation

In the metabolic pathway leading to metabolite Z, an amino acid is converted to an enamine/imine intermediate via a PLP- or FAD-dependent enzyme (Enz). The intermediate has multiple possible fates. The intermediate may diffuse throughout the cell and interact with other components of the metabolic network or is spontaneously deaminated spontaneously by water to form a 2-ketoacid. In certain cases, this reaction is facilitated by a Rid deaminase. Alternatively, evidence suggests certain metabolic network configurations exist that can quench reactive intermediates, and “Y” represents this of possible interaction.

APPENDIX A

SALMONELLA ENTERICA RIDA STRAINS ARE SENSITIVE TO PERTURBATIONS IN ARGININE/PYRIMIDINE METABOLISM

A.1 INTRODUCTION

The pleiotropic phenotypes of a *S. enterica ridA* strain manifest as a consequence of 2-aminoacrylate (2AA) accumulation (1-4). These phenotypes were uncovered and dissected through nutritional supplementation and indicated that 2AA had damaged pyridoxal 5'-phosphate (PLP)-dependent enzymes. Previously, a bioinformatics analysis probing synteny in bacterial genomes predicted associations between RidA and i) PLP-enzymes, ii) FAD-dependent enzymes, and iii) enzymes involved in carbamoyl-phosphate (CP) metabolism (5). The connection to CP was investigated by constructing mutant *Salmonella enterica* strains with altered flux through the branches of carbamoyl-phosphate metabolism (Figure A.1) (6). Introducing a mutation in *argI* (encoding ornithine carbamoyltransferase EC 2.1.3.3) combined with expression of *carB* (encoding carbamoyl phosphate synthase (glutamine hydrolyzing), large subunit EC 6.3.5.5) (7) impeded growth in a *ridA* background (5). Since *S. enterica* withstands these changes in CP flux with a wild type *ridA* allele, the growth defect suggested that additional pathways beyond the CP node might be affected in a *ridA* mutant. These observations strengthened the association between RidA and carbamoyl-phosphate metabolism, but did not address whether 2AA was implicated in eliciting this *ridA* mutant phenotype. This appendix outlines experiments that further probed the association between RidA and carbamoyl-phosphate metabolism.

A.2 MATERIALS AND METHODS

Bacterial strains and media

All strains were *Salmonella Typhimurium* LT2 derivatives and are described in Table A.1. Nutrient broth was used as a rich medium for overnight cultures and terrific broth (tryptone, 12 g/liter; yeast extract 24 g/liter; glycerol, 4 mL/liter; KH_2PO_4 2.31 g/liter; K_2HPO_4 12.54 g/liter) (8) was used in agar plates for the isolation and characterization of suppressor mutants. The pCA24N-*carB* plasmid was obtained from the Keio collection (9) and was provided by Jorge Escalante-Semerena. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM for plasmid induction. To satisfy auxotrophies, L-arginine was added to 600 μM and uracil was added to 100 μM final concentration. Antibiotics were used at the following concentrations: chloramphenicol, 20 g/liter; tetracycline 20 g/liter. All chemicals were purchased from Sigma-Aldrich (MilliporeSigma), St. Louis, MO. All transductional crosses were done using the high-frequency generalized transducing mutant bacteriophage P22 (HT105/1, *int*-201) (10, 11) and standard genetic techniques. Growth analysis was done in liquid culture (5 mL) in borosilicate tubes and was measured via optical density at 650 nm using a Spectronic-20. Growth curves were generated using GraphPad Prism (version 6).

Isolation of suppressors

An overnight culture of DM14351 was spread onto several terrific broth plates containing chloramphenicol and various levels of IPTG. Alternatively, an aliquot (100 μL) of DM14330 overnight culture was added to NCE containing diethyl sulfate (DES) and incubated at 37°C for 1 hour. A sample (100 μL) was used to inoculate NB (5 mL) and grown overnight. The resulting culture was transformed with pCA24N-*carB* and colonies that grew on NB plates containing chloramphenicol were screened for the ability to grow in NB containing 0.1 mM IPTG. Only

strains that retained the ability to grow after curing and re-transforming the strain with a fresh pCA24N-*carB* plasmid were analyzed further. These strains were assigned *cps* allele numbers (*cps1*, 10, 11, and 15) for carbamoyl-phosphate suppressor and are listed in Table A.1. *cps1* was a spontaneous suppressor and *cps10*, 11, and 15 were isolated following DES mutagenesis.

An attempt was made to map the *cps1* suppressor mutation via a Tn10 transposon hop and characterization of strains with transposons linked to the *cps1* locus. Three linked insertions were identified and pursued (*zxx10200::Tn10*, *zxx10201::Tn10*, and *zxx10202::Tn10*). Pairs of strains isogenic at the *cps* locus were re-transformed with pCA24N-*carB* and growth was tested in NB (5 mL) containing chloramphenicol and 0.1 mM IPTG. Of these three isogenic pairs, only the *zxx10201* strain continued to suppress the *ridA* mutant phenotype upon reconstruction.

Degenerate sequencing with transposon-specific primers (Tn10R 5'-

ACCTTTGGTCACCAACGCTTTTCC -3'

and Tn10L 5'- TCCATTGCTGTTGACAAAGGGAAT -3') and arbitrary primers (Arb1 5'-

GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT -3' and Arb6 5'-

GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC -3') was used to sequence out of the transposon, and indicated the insertion had been made near the *lacZ* gene of the MudJ in the *ridA* locus.

A.3 RESULTS AND DISCUSSION

***A Salmonella enterica ridA pyrB* strain is sensitive to increased carbamoyl-phosphate**

Carbamoyl phosphate (CP) is a precursor for both L-arginine and uracil (pyrimidines) (12-15). Based on the relative *ridA* growth defect elicited by a block in arginine biosynthesis (*argI*), it was reasonable to expect that a mutation affecting the uracil branch could similarly compromise growth when *carB* is expressed. A *pyrB* (encoding aspartate carbamoyltransferase

EC 2.1.2.3) mutation was introduced into a *ridA* mutant strain. Relevant strains were transformed with pCA24N-*carB* to generate DM15208 (*pyrB ridA*) and DM15209 (*pyrB*). Growth in NB was monitored without and with induction (0.1 mM IPTG) at 42°C and the data are shown in Figure A.2. As predicted, a *ridA* mutation in a *pyrB* background resulted in a growth defect when *carB* was expressed. However, the *pyrB ridA* strain grew better than the *argI ridA* strain under similar conditions (Figure A.2). These data indicated that a block in either arginine biosynthesis (*argI*) or pyrimidine biosynthesis (*pyrB*) was sufficient to affect growth of a *ridA* strain with increased CP production. It is possible that a greater proportion of CP is directed toward the arginine branch in comparison to the pyrimidine branch, which might explain why a mutation in *argI* led to a more severe growth defect in a *ridA* strain than a mutation in *pyrB*.

Conditions that alleviate *ridA* sensitivity to CP perturbations

The sensitivity of a *ridA* mutant strain to perturbations in CP metabolism was first observed in rich medium (NB) at 42°C (5). The relative growth defect of a *ridA argI* strain expressing *carB* was slight when grown in minimal glucose medium at this temperature (data not shown) in contrast to the growth defect in rich medium. This suggested that the *ridA* growth defect was not likely due to a nutritional limitation under the conditions tested. Any conditions that improve growth of the *ridA* mutant could potentially uncover areas of metabolism affected by the change in CP flux and would provide a platform to elucidate whether RidA plays a role.

The effect of temperature upon growth was explored. Two strains containing the pCA24N-*carB* plasmid, DM14200 (*argI ridA*) and DM14203 (*argI*) were grown in NB with 0.1 mM IPTG at three temperatures: 30°C, 37°C, or 42°C (Figure A.3 Panels A-C). After 10 hours of growth at 30°C, the optical density of the *argI ridA* mutant strain matched the growth of a strain with a wild type *ridA* locus. The two conditions that improved growth of a *ridA* with

altered CP flux were i) growth in minimal medium and ii) growth at 30°C. Both scenarios would slow the bacterial growth rate in comparison to growth in rich medium at 42°C. Based on these growth phenotypes, it is plausible that the *ridA* mutation might exacerbate a defect in general protein folding, or in the heat shock response when coupled to altered CP metabolism.

In order to address whether the *ridA* mutation was causative, a complementation experiment was designed. As CP manipulations were provided via expression of the pCA24N-*carB* plasmid with a modified pMB1 origin of replication, (9) a compatible plasmid was required. Unexpectedly, introduction of a compatible pBAD30 (p15A origin of replication) (16) plasmid control rescued growth of the *argI ridA*/pCA24N-*carB* strain growing in rich medium with 0.1 mM IPTG. Therefore, it was not possible to interpret the effect of providing *ridA* *in trans*. This prompted a closer examination of additional mutant *ridA* alleles to test causality. Possible solutions for future complementation efforts include a single-plasmid system such as a pETDuet-1 vector that would express *carB* as well as *ridA*. Alternatively, an approach may be designed with a chromosomal construct for *carB* expression similar to the inducible *ilvA219* construct that has been described (17).

Particular *ridA* mutants tolerate carbamoyl-phosphate metabolism perturbations

Multiple *ridA* alleles have been isolated that are sensitive to serine and therefore were designated null mutations (18, 19). In contrast to uniform serine sensitivity, differences arose between the *ridA* alleles when introduced into backgrounds with altered CP metabolism. Isogenic strains containing the various *ridA* alleles were constructed in an *argI pyrB* (DM14339) background via transduction with P22 phage lysate grown on the *ridA* donor strains. Uracil prototrophs were selected on minimal glucose medium containing arginine. Resulting strains were confirmed to be arginine auxotrophs and sensitive to serine.

The *argI ridA* strains were transformed with pCA24N-*carB* and grown in NB containing chloramphenicol and 0.1 mM IPTG. The relative growth of each *ridA* strain is indicated in Table A.2. Surprisingly, under conditions that prevented growth of a *ridA3::MudJ* strain, *ridA6*, 7, 8, 10, 24-28, and 33 mutants were indistinguishable from a similar strain with wild type *ridA* locus. In an *argI* background, a *ridA1::Tn10* strain had intermediate growth under similar conditions. These results raised a question as to whether another entity encoded by the transposons was responsible for this growth effect rather than the loss of RidA function.

The possibility that an element of the Tn10 or MudJ transposons was responsible for the mutant phenotype was minimized by two observations. First a MudJ transposon was inserted elsewhere in the chromosome (*purC*) of a Δ *argI2401 ridA::Tn10* strain and did not induce sensitivity to *carB* expression. Second, an *argI::Tn10* strain is not sensitive to CP perturbations except in combination with the *ridA3::MudJ* allele. It is possible that the *ridA6*, 7, 8, 10, 24-28, and 33 alleles produced inactive or improperly folded proteins, and that active RidA protein may not be required to withstand the perturbations to CP metabolism.

Suppressor characterization

In order to identify the areas of metabolism affected by perturbations to CP in a *ridA* background, suppressors were isolated spontaneously on plates or in liquid medium following diethyl sulfate (DES) mutagenesis. Four suppressors were isolated (*cps1*, 10, 11, 15) that restored growth in an *argI ridA* background when transformed with a fresh pCA24N-*carB*. This suggested that the putative suppressor mutations were likely in the chromosome, in contrast to a plasmid mutation that would result in lower *carB* expression. The locations of the suppressor mutations remain unknown. A linked Tn10 was placed near the *cps1* locus and degenerative

sequencing indicated that the transposon was inserted within the *lacZ* gene of the *ridA3::MudJ* locus and suggests the *cps1* mutation is located within ~40 kb on either side of the *ridA* gene.

Conclusions

RidA has been implicated in diverse processes in bacteria due to the mutant phenotypes that arise in its absence. Ultimately all of the growth defects likely stem from 2AA accumulation either directly or indirectly. In considering the selective pressure that maintained *ridA* in all domains of life and in observing the proximity of *ridA* to carbamoyl-phosphate metabolism genes in multiple organisms, it was reasonable to question whether RidA was connected to any of the enzymes involved in CP metabolism. While it is possible to generate genetic scenarios that perturb CP metabolism and prevent growth specifically in a *ridA* strain, it is not clear whether the growth defect is due to 2AA and therefore reflective of the broad damage caused by this reactive metabolite. Any additional experimentation on the potential connections between RidA and CP metabolism should first probe whether the *ridA* mutation is causative of the described growth defects. If the *ridA* mutation is causative, the next step will be to determine whether active RidA is required. Once these questions are addressed, it may prove to be an exciting area in which to probe metabolic integration and the role of *S. enterica* RidA.

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A.4 REFERENCES

1. Flynn JM, Christopherson MR, Downs DM. Decreased coenzyme A levels in *ridA* mutant strains of *Salmonella enterica* result from inactivated serine hydroxymethyltransferase. *Mol Microbiol.* 2013;89(4):751-9.
2. Flynn JM, Downs DM. In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5'-phosphate cofactor. *J Bacteriol.* 2013;195(16):3603-9.

3. Ernst DC, Downs DM, Metcalf WW. 2-Aminoacrylate Stress Induces a Context-Dependent Glycine Requirement in *iridA* Strains of *Salmonella enterica*. *Journal of Bacteriology*. 2016;198(3):536-43.
4. Lambrecht JA, Flynn JM, Downs DM. Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions. *J Biol Chem*. 2012;287(5):3454-61.
5. Niehaus TD, Gerdes S, Hodge-Hanson K, Zhukov A, Cooper AJ, ElBadawi-Sidhu M, et al. Genomic and experimental evidence for multiple metabolic functions in the *RidA/YjgF/YER057c/UK114 (Rid)* protein family. *BMC Genomics*. 2015;16:382.
6. Jenness DD, Schachman HK. *pryB* mutations as suppressors of arginine auxotrophy in *Salmonella typhimurium*. *J Bacteriol*. 1980;141(1):33-40.
7. Nyunoya H, Lusty CJ. The *carB* gene of *Escherichia coli*: a duplicated gene coding for the large subunit of carbamoyl-phosphate synthetase. *Proc Natl Acad Sci U S A*. 1983;80(15):4629-33.
8. Protocols CSH. Terrific Broth 2015 8-3-17 [cited 2017:[1 p.].
9. Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, et al. Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res*. 2005;12(5):291-9.
10. Schmieger H. Phage P22-mutants with increased or decreased transduction abilities. *Mol Gen Genet*. 1972;119(1):75-88.
11. Downs DM, Petersen L. *apbA*, a new genetic locus involved in thiamine biosynthesis in *Salmonella typhimurium*. (0021-9193 (Print)).
12. Navre M, Schachman HK. Synthesis of aspartate transcarbamoylase in *Escherichia coli*: transcriptional regulation of the *pyrB-pyrI* operon. *Proc Natl Acad Sci U S A*. 1983;80(5):1207-11.
13. Legrain C, Stalon V, Glansdorff N, Gigot D, Pieard A, Crabeel M. Structural and regulatory mutations allowing utilization of citrulline or carbamoylaspartate as a source of carbamoylphosphate in *Escherichia coli* K-12. *J Bacteriol*. 1976;128(1):39-48.
14. Schwartz M, Neuhaud J. Control of expression of the *pyr* genes in *Salmonella typhimurium*: effects of variations in uridine and cytidine nucleotide pools. *J Bacteriol*. 1975;121(3):814-22.
15. Cunin R, Glansdorff N, Pierard A, Stalon V. Biosynthesis and metabolism of arginine in bacteria. *Microbiol Rev*. 1986;50(3):314-52.

16. Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol.* 1995;177(14):4121-30.
17. Borchert AJ, Downs DM. The Response to 2-Aminoacrylate Differs in *Escherichia coli* and *Salmonella enterica*, despite Shared Metabolic Components. *J Bacteriol.* 2017;199(14).
18. Enos-Berlage JLL, Mark J.; Downs, Diana M. Complex metabolic phenotypes caused by a mutation in *yjgF* encoding a member of the highly conserved YER057c/YjgF family of proteins. *Journal of Bacteriology.* 1998;180(24):6519-28.
19. Schmitz G, Downs DM. 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. *Journal of Bacteriology.* 2004;186(3):803-10.
20. Castilho BA, Olfson P, Casadaban MJ. Plasmid insertion mutagenesis and lac gene fusion with mini-mu bacteriophage transposons. *J Bacteriol.* 1984;158(2):488-95.
21. Way JC, Davis MA, Morisato D, Roberts DE, Kleckner N. New Tn10 derivatives for transposon mutagenesis and for construction of lacZ operon fusions by transposition. *Gene.* 1984;32(3):369-79.
22. Bochner BR, Huang HC, Schieven GL, Ames BN. Positive selection for loss of tetracycline resistance. *J Bacteriol.* 1980;143(2):926-33.
23. Maloy SR, Nunn WD. Selection for loss of tetracycline resistance by *Escherichia coli*. *J Bacteriol.* 1981;145(2):1110-1.

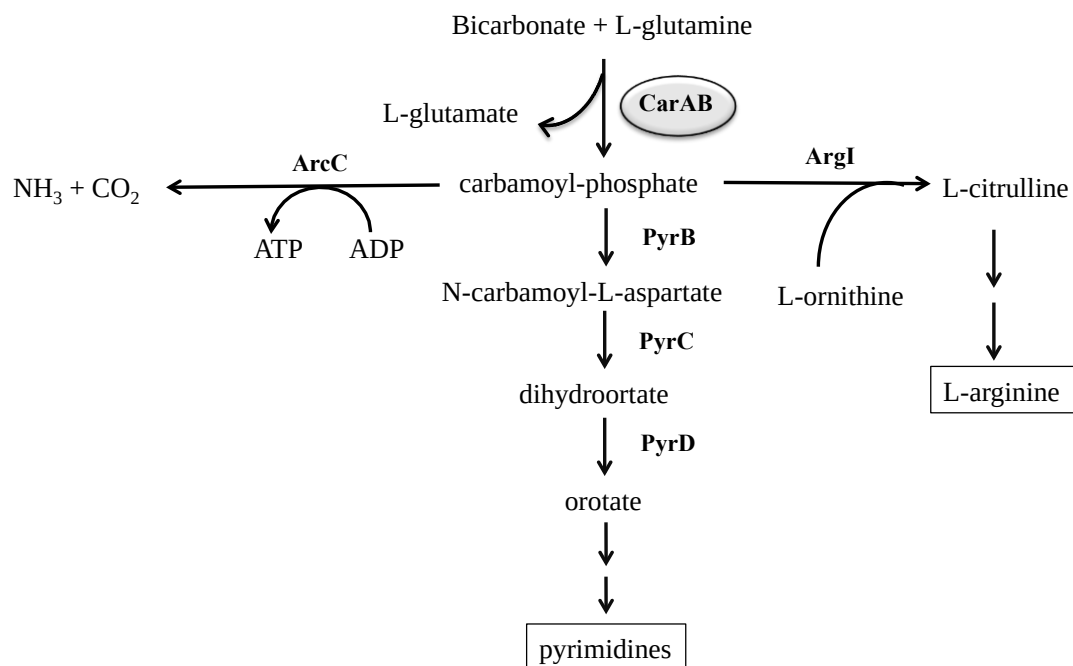


FIGURE A.1 Carbamoyl-phosphate metabolism in *Salmonella enterica*. Carbamoyl-phosphate synthase (CarAB) is generates carbamoyl phosphate, which is the branching point for ammonia and ATP production or biosynthesis of pyrimidines or L-arginine.

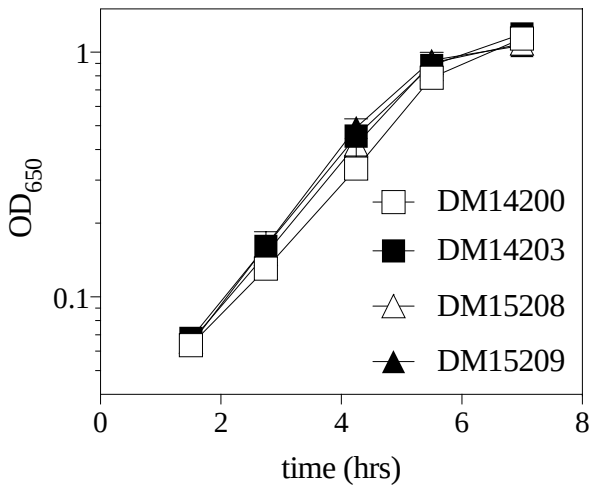
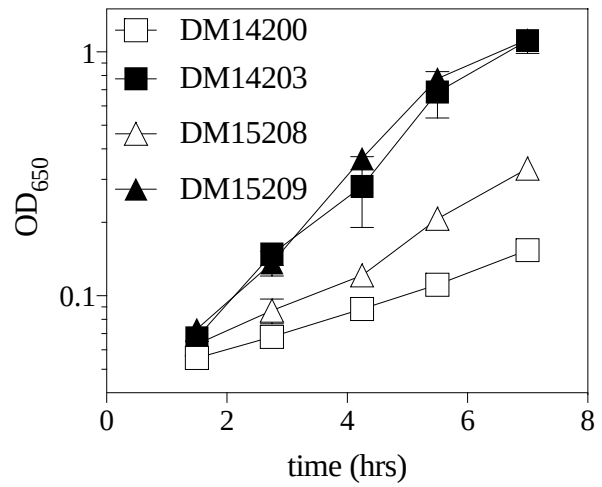
A**B**

FIGURE A.2 A *ridA* strain has a growth defect in either *pyrB* or *argI* backgrounds

Strains harboring the pCA24N-*carB* plasmid were grown in NB (5 mL) containing chloramphenicol at 42°C without (A) or with (B) 0.1 mM IPTG. Strain numbers and relevant genotypes are: DM14200 (*argI ridA*), DM14203 (*argI*), DM15208 (*pyrB ridA*), and DM15209 (*pyrB*).

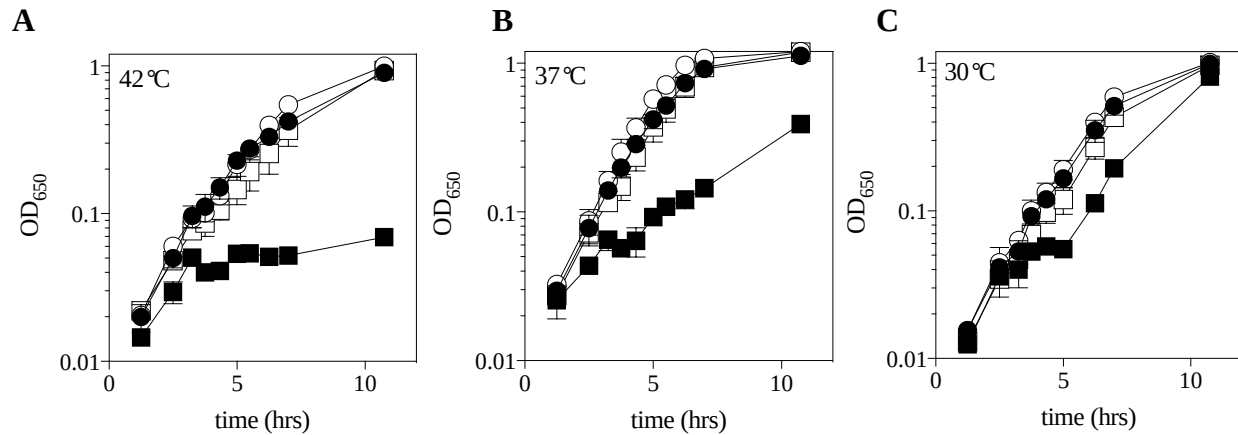


FIGURE A.3 Growth of *ridA* strains is improved at lower temperatures.

S. enterica strains harboring pCA24N-*carB* were grown in NB (5 mL) containing chloramphenicol at (A) 42°C, (B) 37°C, or (C) 30°C with shaking. Strain numbers, relevant genotypes, and symbols used in the graphs above are as follows: DM14200 (*argI ridA*, squares) and DM14203 (*argI*, circles). Strains were grown in with (closed symbols) or without (open symbols) the presence of 0.5 mM IPTG for the induction of the plasmid.

TABLE A.1 Strains used.

Strain	Relevant genotype	References
DM14200	<i>argI833:Tn10 ridA3::MudJ^a/ pCA24N-carB</i>	(5)
DM14203	<i>argI833:Tn10/ pCA24N-carB</i>	(5)
DM14330	<i>ΔargI2401 ridA3::MudJ</i>	This study
DM14339	<i>ΔargI2401^c pyrB692::Tn10d(Tc)^b</i>	This study
DM14351	<i>ΔargI2401 ridA3::MudJ/ pCA24N-carB</i>	This study
DM15170	<i>ΔargI2401 ridA3::MudJ zxx10200::Tn10 cps1</i>	This study
DM15172	<i>ΔargI2401 ridA3::MudJ zxx10201::Tn10 cps1</i>	This study
DM15174	<i>ΔargI2401 ridA3::MudJ zxx10202::Tn10 cps1</i>	This study
DM15259	<i>ΔargI2401 ridA3::MudJ cps10</i>	This study
DM15261	<i>ΔargI2401 ridA3::MudJ cps11</i>	This study
DM15263	<i>ΔargI2401 ridA3::MudJ cps15</i>	This study
DM15208	<i>pyrB692::Tn10d(Tc) ridA3::MudJ/ pCA24N-carB</i>	This study
DM15209	<i>pyrB692::Tn10d(Tc) /pCA24N-carB</i>	This study
DM15278	<i>argI833:Tn10 ridA7</i>	This study
DM15358	<i>argI833:Tn10 ridA6</i>	This study
DM15359	<i>argI833:Tn10 ridA8</i>	This study
DM15360	<i>argI833:Tn10 ridA10</i>	This study
DM15364	<i>argI833:Tn10 ridA33</i>	This study
DM15365	<i>argI833:Tn10 ridA24-28</i>	This study
DM15373	<i>ΔargI2401 ridA1::Tn10</i>	This study

^aMudJ refers to the Mud dl1734 transposon (20)

^bTn10d refers to the transposition-defective mini-Tn10 (Tn10Δ-16 Δ-17) (21)

^c *ΔargI2401* was isolated by the Bochner method of selection for the loss of tetracycline resistance (22, 23)

TABLE A.2 Mutant *ridA* alleles tolerate carbamoyl-phosphate production differently

Relevant Genotype	Growth when pCA24N- <i>carB</i> is induced ^a
<i>ridA1::Tn10</i> ^b	-
<i>ridA3::MudJ</i>	-
<i>ridA6</i>	+++
<i>ridA7</i>	+
<i>ridA8</i>	+++
<i>ridA10</i>	+++
<i>ridA24-28</i>	+++
<i>ridA33</i>	+++

^aGrowth symbols indicate: growth is comparable to a wild type *ridA* locus (+++), growth was intermediate (+), or growth was inhibited (-). Cultures were grown in NB/Cm20 (5 mL) medium and grown at 42°C with shaking. The pCA24N-*carB* plasmid was induced with 0.1 mM IPTG.

^b*ridA1::Tn10* strain was constructed in an $\Delta argI2401$ background to yield strain DM15373. All other alleles were constructed in *argI833::Tn10* background.