INVESTIGATING RIDA ENZYMES OF *PSEUDOMONAS AERUGINOSA* AND *CAMPYLOBACTER JEJUNI*

by

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(Under the Direction of Diana Downs)

ABSTRACT

The Rid (YjgF/YER057c/UK114) protein superfamily is conserved in all domains of life and members of the family have been implicated in a myriad of metabolic processes. The physiological role and conserved biochemical activity of RidA (reactive intermediate deaminase), the archetypal member of the protein family, was demonstrated using biochemistry and genetics in *Salmonella enterica*. The RidA paradigm established in *S. enterica,* serves as a framework to study the integration of RidA into the metabolic networks of other organisms. Mutant phenotypes arise in multiple organisms, both eukaryotic and prokaryotic, that lack RidA and evidence suggests damage by the reactive metabolite 2-aminoacrylate (2AA) is responsible for these phenotypes. The reactive metabolite, 2AA, is generated by some pyridoxal 5'-phosphate (PLP)-dependent enzymes including serine/threonine dehydratase, cysteine desulfhydrase, and diaminopropionate lyase in *S. enterica.* In the absence of RidA, 2AA accumulates and damages other PLP-dependent enzymes, which leads to diverse phenotypes depending on the unique metabolic network of the organism.

The research that follows aimed to expand understanding RidA integration into diverse metabolic networks using *Pseudomonas aeruginosa* and *Campylobacter jejuni* as model organisms. When compared to the established paradigm from *S. enterica,* the data show similarities and differences in the metabolic integration of RidA in *P. aeruginosa* and *C. jejuni.* Like *S. enterica*, *P. aeruginosa* and *C. jejuni* encode multiple Rid enzymes, yet removing only one was sufficient to elicit metabolic defects. *S. enterica, P. aeruginosa* and *C. jejuni ridA* mutants have motility defects, suggesting the impact of 2AA accumulation is far reaching despite metabolic differences. *P. aeruginosa* and *S. enterica* share the major route of 2AA accumulation through the biosynthetic serine/threonine dehydratase, but the critically damaged enzymes differ in each organism. In *C. jejuni* the biosynthetic serine/threonine dehydratase was not the generator of 2AA under the conditions tested. In total, this work expanded understanding of the RidA paradigm and gave insight into the unique metabolic networks *P. aeruginosa* and *C. jejuni.*

INDEX WORDS: 2-aminoacrylate, Metabolic stress, Pyridoxal 5'-phosphate, RidA

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DEDICATION

This thesis is dedicated to all of the graduate students that follow. You are the foundation of scientific advancement. Keep going!

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CHAPTER 1

INTRODUCTION

1.1 Introduction to RidA.

The decreased cost and ease of whole-genome sequencing has led to an enormous increase in sequencing analyses over the last two decades. These data have provided researchers with the opportunity to explore the metabolic content of diverse organisms by identifying component parts. While the function of some gene products is defined experimentally, as sequence data has outpaced experimental capacity, we have become more dependent on computation to classify and categorize the genome content. Computational analysis assigns function and, thus, annotation, to proteins of unknown function by i) identifying homologous proteins from other available sequences and databases and ii) inferring function by comparing functions of homologs and regions of homology that are shared with the novel protein (1). In total, analyses of genomes have revealed that biological components and principles are largely conserved across diverse organisms. This realization led to the idea that protein function can be predicted by protein sequence and homology of protein motifs alone. Though, computational annotation has significant limitations as rates of experimental evidence for each annotation lag far behind the rate of annotation, annotations are often assigned with minimal protein sequence similarity leading to overprediction, annotations differ depending on which database was used, and misannotations proliferate throughout time despite lack or addition of experimental evidence (2).

Levels of misannotation have increased as high throughput sequencing technologies have advanced and can be attributed to overprediction of protein function and propagation of

misannotation over time and among databases (2). Around one-third of the protein-coding genes in the bacterial genome are of unknown function, computational misannotation becomes more common as sequences amass, and misannotation may impede valuable data analysis. In the -omics era of biology, it is crucial that we continue to rely on established experimental approaches to elucidate functions of novel proteins. Classical molecular and genetic approaches allow an unbiased means to uncover a protein function, whereas, protein sequence homology can be misleading and occlude data that support novel, and/or unexpected, protein functions/enzyme activities (3, 4). Further, sequence prediction of a metabolic network alone is not sufficient to predict the integration of a specific protein or metabolite into the metabolic architecture of an organism. The specific metabolic architecture of each diverse organism influences the systemic response to perturbations of the network.

The work that follows builds on the RidA paradigm. RidA (reactive intermediate deaminase A) is an ancient and critical enzyme found in all domains of life that mediates damage to enzymes that use the cofactor, pyridoxal 5'-phosphate (PLP). Early studies of RidA from mammalian liver reported RidA proteins shared sequence homology to heat-shock or chaperone proteins and purified with chromatin proteins (5, 6). These studies ostensibly informed the role of RidA homologs for almost two decades until a conserved biochemical function was determined (7). Despite rigorous work using classical genetics and biochemistry to define the conserved deaminase activity of RidA enzymes from all domains of life, in present-day literature, RidA enzymes are frequently misannotated as endoribonucleases, hypothetical or heat-shock proteins. Similarly, amino acid residues previously demonstrated to be catalytically critical are frequently ignored. In this work, I've begun to address the role of conserved enzymes in the context of diverse metabolic networks to i) expand the understanding of the established RidA paradigm, ii) generate

fundamental insights into metabolism of diverse organisms and iii) understand how reactive metabolites can perturb complex systems in different ways depending on the unique metabolic architecture of the organism.

The RidA stress paradigm was discovered using an unbiased genetic approach in *Salmonella enterica* and revealed an uncharacterized endogenous metabolic stress and a unique enzyme function usually ascribed to a spontaneous reaction involving water (8, 9). RidA deaminates 2-aminoacrylate (2AA), a toxic intermediate in certain metabolic reactions. In the absence of RidA, 2AA accumulates and can damage specific PLP-dependent enzymes, perturbing the metabolic network. There are essential members of the PLP-dependent family of enzymes that participate in various metabolic pathways throughout the domains of life, highlighting the importance of preempting damage to these enzymes across species. The novel function of RidA in mediating endogenous metabolic stress may have been overlooked if studies relied on protein sequence homology alone, without use of unbiased genetics.

RidA enzymes from diverse organisms have been studied in and outside of their unique metabolic contexts. *In vivo* experiments using bacteria, yeast, and plant RidA enzymes suggest a conserved framework for the RidA stress paradigm where 2AA accumulates in a *ridA* mutant and damages some PLP-dependent enzymes leading to metabolic defects. Integration of RidA into the distinct metabolic network of each organism results in varying phenotypic outputs. The generators of 2AA and the critically damaged enzymes depend on the distinct metabolic architecture of the organism. Here we expand the RidA paradigm that was defined in *S. enterica* to include *Pseudomonas aeruginosa* and *Campylobacter jejuni*.

1.2 Dissertation outline

The dissertation that follows outlines my efforts to expand understanding of the RidA stress paradigm in two distinct organisms, *P. aeruginosa* and *C. jejuni*, and to define the 2AA generators, targets, and phenotypic consequences of removing *ridA* from each metabolic network. *P. aeruginosa* is an opportunistic and aerobic pathogen with a large genome (6.4 Mb) capable of colonizing varied environments such as lung tissue, skin lesions, the gastrointestinal track, and medical devices (e.g. catheters). *C. jejuni* is a gastrointestinal and microaerophilic pathogen with a small genome (1.6 Mb) that is capable of persisting in the digestive tracks of mammals and birds, raw meat, water, and milk. Despite the dissimilarities, *ridA* mutants of *P. aeruginosa* and *C. jejuni* have motility defects, similar to an *S. enterica ridA* mutant, suggesting the consequences of 2AA accumulation have far-reaching effects despite metabolic differences (10-12). The integration of RidA into the metabolic networks appears to differ, reflecting the diversity of each organism.

Chapter 2 integrates findings from almost three decades of RidA-related research spanning all domains of life. I hope this document will serve as a resource for researchers across fields to gain insights into the breadth and depth of RidA studies, thwart the propagation of misannotation of RidA enzymes, encourage investigation of RidA enzymes to include the conserved biochemical activity, and urge construction of knockout mutants and mutations in amino acid residues critical for the conserved deaminase activity of RidA enzymes. This literature review covers activities assigned to RidA enzymes aside from the conserved deaminase activity, the discovery of RidA deaminase activity, the physiological consequences of removing RidA from the *S. enterica* and other metabolic networks, and recommended experiments to address the role of RidA in diverse organisms.

In chapter 3, I describe initial studies to explore the role of RidA in the unique metabolic context of *C. jejuni*, an epsilonproteobacterium and gastrointestinal pathogen. *C. jejuni* encodes

two Rid enzymes and an insertion in $_{cj}$*ridA* (*cj*1388) led to defects in flagellar biosynthesis and/or function that was confirmed by transmission electron microscopy. The phenotypes of a $_{cj}$*ridA* mutant, suggested 2AA accumulates and damages PLP-dependent enzymes in *C. jejnui*. However, the generators, targets, and consequences of 2AA accumulation in *C. jejuni* differ from *S. enterica* and *P. aeruginosa.*

Chapter 4 describes work to understand the integration of PA5339, $_{PA}$RidA, into the metabolic network of *P. aeruginosa. P. aeruginosa* encodes eight Rid proteins and only an insertion in a single Rid coding sequence, *PA5339*, lead to a discernable phenotype under the conditions tested. This work demonstrated the growth, motility, and biofilm formation defects of a *P. aeruginosa* $_{pa}$*ridA* mutant and confirmed PA5339 is a genuine RidA enzyme *in vivo* and *in vitro*. The phenotypes of the $_{pa}$*ridA* mutant suggest a partially conserved framework for the RidA stress paradigm in *P. aeruginosa* where 2AA is generated from serine/threonine dehydratase (IlvA), leaves the enzyme active site and damages specific PLP-dependent enzymes. However, the critical PLP-dependent enzyme targets of 2AA are not conserved between in *P. aeruginosa* and *S. enterica.*

Chapter 5 describes the state of my efforts to characterize mutants that suppress serine sensitivity and/or motility defects in the *P. aeruginosa* $_{pa}$*ridA* mutant. Whole-genome sequencing was used to identify a single nucleotide polymorphism in each suppressor mutant and mutants were phenotypically characterized. This work uncovered valine and leucine sensitivities of *ridA* mutants and additional metabolic connections to RidA in *P. aeruginosa.*

In chapter 6, I summarize conclusions for my work and outline directions for future studies to expand the understanding of the RidA paradigm in *P. aeruginosa* and *C. jejuni.*

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CHAPTER 2

THE RIDA SUPERFAMILY CONTROLS ENAMINE STRESS IN MICROBES

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2.1 Summary

The Rid protein superfamily (previously YjgF/YER057c/UK114) contains eight subfamilies, (RidA, and Rid1-7) (1). The RidA subfamily is found in all domains of life, with the vast majority of free-living organisms carrying at least one RidA homolog. The remaining subfamilies, Rid1-7, are broadly distributed and exist solely in prokaryotes. In over two decades, around 100 reports have implicated Rid protein family members in cellular processes in prokaryotes, yeast, plants, and mammals. Functional roles have been proposed for RidA enzymes in amino acid biosynthesis, plant root development and nutrient acquisition, cellular respiration, and carcinogenesis. RidA proteins are small and trimeric, tend to be heat stable, are abundant in the mammalian liver, have antiviral properties, are surface exposed on some bacterial pathogens and cancer cells, and are pan-allergens. Despite the wealth of literature and over a dozen high resolution structures of different RidA enzymes, the biochemical function of these proteins remained elusive for decades. Enamine deaminase activity was attributed to RidA proteins in 2012 as a consequence of biochemical and genetic studies in *Salmonella enterica.* This activity has been conserved in RidA enzymes across all domains. Work subsequent to the identification of biochemical activity described the RidA paradigm in *S. enterica* and attributed the phenotypes of mutants lacking RidA to the accumulation of the reactive metabolite 2-aminoacrylate (2AA) and resulting damage to metabolic enzymes. Conservation of enamine deaminase activity in RidA enzymes from all domains raises the likelihood that, despite the diverse phenotypes, the consequences of lacking RidA are due to accumulated 2AA (or a structurally similar enamine) and the diversity of metabolic phenotypes can be attributed to differences in metabolic network architecture. This review focuses primarily on the RidA subfamily (Reactive intermediate deaminase A), describes experiments that led to elucidation of its conserved biochemical activity

and physiological role in the metabolic context of *S. enterica.* The Rid paradigm in other organisms and the status of our understanding of the Rid1-7 subfamilies is also discussed. Finally, this review discusses fundamental metabolic lessons learned from the RidA paradigm in microbes, implications for metabolic network evolution and diversity, and recommendations on how to address key questions about this widely conserved family of proteins.

2.2 Introduction

The YjgF/YER057c/UK114 (Rid) superfamily was first defined by sequence homology using 80 protein sequences of bacteria, archaea, plants, and eukaryotes (2). Since that designation, members of this superfamily have been found in the majority of sequenced genomes (1). The presence of Rid family members in all domains of life suggested an ancient and essential role for these enzymes. Enzymes that belong to this family have been referenced in the literature for more than 25 years, many of them before a biochemical activity was rigorously demonstrated. Throughout these reports, YjgF/YER057c/UK114 proteins were implicated in numerous biological processes in multiple organisms. A pivotal discovery in the field came from work in *S. enterica* with the *in vivo* and *in vitro* demonstration that homologs across domains of life had a conserved enamine/imine deaminase activity (3). In total this work justified renaming YjgF/YER057c/UK114 protein family, Rid (**r**eactive enamine/**i**mine **d**eaminase). This family was later divided into eight subfamilies (RidA and Rid 1-7) based on phylogenetic grouping by the NCBI Conserved Domain Database (cd00448: YjgF_YER057c_UK114_family). Further analysis of conserved sequences placed the subfamilies into two groups i) RidA and Rid1-3 that had a conserved active site residue, Arg105, and were predicted to have enamine/imine deaminase activity and ii) Rid4-7 that lacked the critical active site residue and were predicted to catalyze

other reactions (1). The pattern of the Rid family phylogenetic distribution suggested the RidA subfamily is the archetypal member that gave rise to other subfamilies (1).

The discovery of the RidA paradigm in *S. enterica* laid a foundation for assessing the role of Rid proteins in diverse organisms. This review describes the studies that defined the conserved function of RidA, the paradigm of enamine stress in *S. enterica*, and emerging studies that explore how this paradigm differs in other organisms. Further, we discuss other functions that have been suggested and/or demonstrated for Rid proteins, describe the current status of the field, and pose questions that will drive future studies.

2.3 Diverse roles assigned to YjgF/YER057c/UK114 proteins

For over two decades, members of the YjgF/YER057c/UK114 protein family, designated RidA herein, have been reported to have diverse functional roles. The earliest recognized reference to a member of the Rid protein superfamily was a 1993 study of chromatin-associated proteins. This RidA protein was isolated from rat liver based on its unusual solubility in perchloric acid (4). The RidA protein was unnamed in this study and was classified as a small ~23 kDa acidic dimer under the conditions tested (*S. enterica* RidA was found to have similar solubility in acid, Enos-Berlage and Downs, unpublished). This early study referenced protein sequence alignments (not shown) used to assign the RidA a "heat-shock" or chaperone designation based on 18% to 27% identity to a motif from large heat-shock proteins (82-90 kDa) from humans, mouse, fruit fly and some single-celled organisms. The designation as a heat-shock protein, or chaperone, was propagated throughout the literature since the early 1990's, has not been demonstrated repeatably, and has not been shown to be physiologically relevant (discussed in section 2.20) (5-8). Prior to the definitive characterization of a biochemical activity for RidA proteins across domains of life in 2012 (9), several publications suggested a variety of activities for RidA proteins such as

ribonuclease and/or general translation inhibitor (5, 6, 10, 11), aromatic degradation protein (12-14), calpain activator (15), regulator of purine synthesis (16), fatty acid binding protein (17), and heat-shock protein and/or chaperone (18). RidA proteins were found localized to plant chloroplasts; rat liver, kidney, intestines and lung tissues; and human monocytes, all of which suggests a physiologically relevant role for RidA proteins that remains to be explored (4-7, 19-22). To date, none of the activities assigned to RidA proteins, other than the conserved enamine deaminase activity (discussed section 2.7), were confirmed to be physiologically relevant. The several key questions remain, do any of the assigned functions of RidA rely on the conserved biochemical activity of the enzymes; are the varied assigned functions physiologically relevant; and does the observed localization of RidA enzymes to specific organs, cell types, or organelles indicate a physiological relevance, and if so, what is it?

2.4 Structural analyses hinted to a functional role of Rid proteins.

The first crystal structure of a Rid family member (RidA from *Escherichia coli*) was obtained in the context of a functional proteomic study, and was reported in 1999 (23). A number of other structures followed and there currently are over twenty crystal structures of bacterial or eukaryotic RidA homologs in the Protein Data Bank (Table 2.1). All crystal structures of RidA protein across every domains of life show a homotrimer with the presence of a chorismate mutase fold type (2, 23-36). It is important to note, some earlier studies of RidA enzymes reported dimeric structures, however, every crystalized RidA has been a homotrimer. The structural conservation among RidA proteins was emphasized in a study that purified and disassociated goat liver (UK114) and human monocyte (p14.5) trimers. The disassociated monomers were mixed to allow heterogenous trimers to reform (30). The study found that both native and chimeric trimers formed, demonstrating the structural similarity between proteins from different organisms and cell types.

Although several residues that line the active site appeared to be conserved in multiple Rid sequences, a single arginine residue is necessary and sufficient to bind the substrate. The Arg105 (Arg107 in UK114/p14.5 and Arg165 in *Arabidopsis thaliana* RidA) hydrogen bonded to ligands observed in crystallization of *E. coli* TdcF and human RidA homologs, hp14.5 (26, 29, 36). A later study using variant proteins indicated that residues Cys107, Tyr17, and Glu120 were dispensable and supported the conclusion that Arg105 alone was critical to the conserved deaminase activity of RidA enzymes (9). Early structural studies found the presumed RidA active site was nestled between subunits and functioned as a cleft where a water molecule was bound in close proximity to a small molecule substrate (2, 29). The water molecule was bound in the active site by amino acid backbones, rather than functional groups (2, 29). This, in part, could explain the residue diversity in the active site pocket of active Rid deaminase enzymes and, if the critical enzyme bond to the substrate occurs at the carboxyl end, the broad substrate specificity of Rid enzymes is plausible.

Crystal structures with ketoacids or amino acids soaked into the active site suggested key interactions. One study focused on intermediates in isoleucine biosynthesis, such as 2-ketobutyrate, and implied RidA enzymes could be involved the conversion of an enamine to ketoacid, though, since this reaction occurs spontaneously with free water, authors determined an enzyme would not be required (2). These structural results become more valuable when the conserved biochemical activity of RidA enzymes was uncovered in the context of the metabolic system of *S. enterica* (9). In *S. enterica,* the short-lived and reactive, enamine/imine of serine was shown to damage pyridoxal' 5 phosphate (PLP)-dependent enzymes and lead to metabolic defects in the absence of RidA and *in vitro*, RidA enzymes increased the rate of ketoacid (3, 9, 37-39).

These findings demonstrate how structural analysis along with genetic and biochemical investigation can work in tandem to uncover the functional roles of unknown proteins.

Despite several structural studies of Rid proteins, unanswered questions remain, such as the substrate specificity (enamine or imine) of RidA, the roles of other seemingly conserved residues in the active site, and the physiological roles and/or substrate specificities of other Rid subfamily members (Rid1-7). Numerous residues are conserved in RidA homologs, yet, only Arg105 disruption leads to abolition of the conserved biochemical activity and perhaps the other conserved residues suggest a functional role that has yet to be determined (9). Structural, genetic, and biochemical approached to understand the role of Rid 1-7 subfamily members could begin to facilitate efforts to understand the unknown function of these proteins.

2.5 Biochemical and genetic approaches in a model organism led to functional insights.

Early studies of the RidA family noted the striking sequence conservation among proteins from diverse species (*S. enterica* YjgF 40% similarity to goat UK114) (37). The substantial conservation of the RidA protein sequence throughout the domains of life suggested the proteins shared a biochemical activity. If RidA enzyme activity was shared across domains, it was expected that disrupting this activity would have different phenotypic consequences due to the diverse metabolic networks of each organism or cell type. The diverse physiological contexts in which RidA enzymes had been described did not lead to a straightforward hypothesis for a conserved molecular mechanism. Rather, the critical insights into the conserved biochemical function and physiological role of RidA (formerly YjgF) were obtained by extensive and rigorous experimentation in *S. enterica* that was not initiated by a focused effort to define function, but by a serendipitous result. In a triumph of forward genetics, the conserved deaminase activity of RidA enzymes was ultimately uncovered through biochemical genetic analyses of thiamine biosynthesis. *S. enterica,*

mutants lacking *purF* (phosphoribosylpyrophosphate amidotransferase, EC 2.4.2.14) and *gnd* (6-phosphogluconate dehydrogenase, EC 1.1.1.44) are unable to synthesize the pyrimidine moiety of thiamine due to the lack of phosphoribosyl amine (PRA) (Figure 2.1A). Null alleles of *ridA* were isolated as suppressors of the thiamine requirement, which allowed the *purF gnd* strain to synthesize sufficient thiamine for growth (40).

In *S. enterica*, PRA is a precursor to purines and thiamine and is the product of PurF (phosphoribosyl pyrophosphate, PRPP, amidotransferase, EC 2.4.2.14). In the absence of *purF*, in specific environmental conditions and/or genetic background, PRA can be made by the non-enzymic combination of ribose 5'-phosphate (R5P) and ammonia (41-43). Suppression by a *ridA* mutation was independent of both of these metabolites, indicating that the absence of RidA triggered a new mechanism to generate PRA (Figure 2.1B) (37, 44, 45 2012 ASC). Surprisingly, these data indicated that the lack of RidA recruited a new pathway. Over several years genetic analyses determined the role of an enzyme from branched chain amino acid biosynthesis (IlvA, EC 4.3.1.19) and one involved in tryptophan biosynthesis (TrpD, EC 4.1.3.27) in the formation of PRA. Data from these efforts led to a model in which the reactive enamine product derived from threonine (aminocrotonate) by IlvA was used by TrpD, in the presence of phosphoribosylpyrophosphate (PRPP), to generate PRA (44, 45). Critical to this model was the fact that isoleucine prevented the thiamine synthesis by feedback inhibiting IlvA, thus eliminating the formation of one of the substrates for the recruited step. Formation of PRA was reconstituted *in vitro* with IlvA, TrpD, threonine and PRPP (45). Importantly, PRA formation is abolished in the presence of RidA (9, 44, 45). Subsequent work showed that in the presence of RidA (*in vivo* or *in vitro*), PRA was not formed by the proceeding mechanism leading to the early prediction that aminocrotonate was a substrate of RidA (45). In studies that followed, genetic, nutritional, and

biochemical analyses led to a biochemically testable model that predicted the combination of TrpD (anthranilate synthase), IlvA (serine/threonine dehydratase) and threonine would generate the precursor to thiamine synthesis, PRA.

2.6 Serendipitous discovery of protein function highlights advantages of unbiased approach.

The serendipitous observation that resulted in the ability to define a biochemical function and physiological role for RidA highlights the advantages of using non-targeted and unbiased approaches to gain insights that cannot be generated *a priori*. This may be particularly true for understanding conserved proteins of unknown function. Targeted approaches that rely on previous gene annotation, may occlude novel and/or unanticipated function, as was the case for RidA. Understanding of the conserved biochemical activity and physiological role for RidA came from an unbiased screen for random mutations that improved PRA formation under specific nutrient conditions and in a particular mutant background (Figure 2.1). The *ridA* null mutant was identified for a role in a well-studied system and a particular genetic background. These studies were not informed by prior literature citing RidA proteins as a translation inhibitor or heat-shock protein. Instead, in this foundational work, *ridA* mutants were screened for responses to amino acids and/or vitamins central to metabolism and found that *ridA* mutants were sensitive to exogenous serine and that sensitivity was abated by exogenous isoleucine. The serine sensitivity and growth restoration with addition of isoleucine in a *ridA* mutant informed testable models of the biochemical function, physiological role of RidA, and foundation of the RidA stress paradigm in *S. enterica* (and other organisms). This approach is in contrast to targeted experimental design, where the details of physiological function and relevance can be missed.

2.7 Definition of conserved deaminase activity – from untargeted approach to novel discover

A functional role for a protein is best demonstrated by a mutant phenotype in its absence. In the absence of a RidA protein, metabolic defects arise in bacteria, plants, and yeast, such as nutrition, mitochondrial maintenance, C1 metabolism (Figure 2.2), indicating that RidA plays a role in diverse cellular processes. However, the conservation of the Rid protein family through all domain of life could not be explained by the strain specific phenotype of a *S. enterica ridA* mutant to impart a positive growth advantage on a thiamine auxotroph. Instead, nutritional analysis showed that *S. enterica* mutants lacking *ridA* were sensitive to serine and the sensitivity could be abated with the addition of isoleucine, a key finding to extrapolating from the biochemical function of RidA to a physiologically relevant role (9, 40). A simple expansion of the model suggested the serine/threonine dehydratase, IlvA (EC 4.3.1.19), serine derived intermediate, 2-aminoacrylate (2AA) was a substrate of RidA, and that it was the deamination of this reactive metabolite that was the physiologically relevant activity of this RidA.

The phenotypic output of mutants lacking *ridA* led to discovery of the biochemical activity of RidA enzymes and helped to define the RidA stress paradigm in *S. enterica*. The biochemical role conserved across domains for the RidA protein family was demonstrated using purified homologs from *S. enterica* (RidA), *Bacillus subtilis* (YabJ), *Homo sapiens* (UK114), *Cucumis sativus* (ChrD), and *Pyrococcus furiosus* (PF0668) and functionally important amino acid residues where determined using *S. enterica* RidA variants (Y17F, R105A, E120K, and E120A) (9). *In vitro*, the RidA homologs increased the rate of hydrolysis of the enamine/imine intermediates generated by IlvA from threonine or serine (9). This result led to the hypothesis that the Rid protein family increased the rate of hydration of enamine/imine intermediates in the low water environment of the cellular milieu, thus preventing endogenous metabolic stress (9).

IlvA is a PLP-dependent enzyme that dehydrates threonine or serine to generate the enamine, 2-aminocrotinate (2AC) or 2AA, respectively, that tautomerizes to the respective imine prior to hydration with aqueous water. Past studies with similar PLP-dependent enzymes defined the catalytic mechanism and characterized the short half-life of the imine/enamine intermediates resulting in the assumption that the final hydration was non-enzymatic and catalyzed by cellular water (46, 47). In a purified system at physiological pH, RidA increased the rate of 2-ketobutyrate formation from IlvA and threonine 2-fold, whereas, as pH increased the effect of RidA on the reaction rate increased to 4 to 5-fold at pH 9.5 (9). These data supported the conclusion that RidA was able to facilitate imine quenching and high pH contributed to stability of the imine/enamine (9, 48, 49). Significantly, the presence of RidA did not change product outputs from IlvA reactions with threonine or serine, indicating that no new reaction was occurring. Ferricyanide was used to determine the enamine/imine deaminase activity of RidA *in vitro* (9). In the absence of RidA, the majority of the ferricyanide was reduced by a reaction with enamine/imine products of IlvA and threonine, however, in the presence of RidA ferricyanide reduction was inhibited 4-fold (9). Further, RidA increased the rate of pyruvate formation from serine, indicating a specificity of at least 3 and 4 carbon imine/enamines.

Based on the *in* vitro activity assays, the *Salmonella* ORF YjgF, was renamed RidA (**r**eactive enamine/**i**mine **d**eaminase A) in 2012. This moniker was adopted for the protein superfamily and also describes the function shared by every RidA homolog tested to date, including *P. furiosus* PF0668 (3), *C. sativus* ChrD (3), *H. sapiens* UK114 (3, 8), *A. thaliana* (50), *Zea mays* (50), *Dermatophagoides farina* (house dust mite) (51), *E. coli* (52), *Pseudomonas aeruginosa* (53), *Campylobacter jejuni* (54), *Yersinia pestis* (Martínez, *in preparation*), and *Saccromyces cerevisae* (55) (Table 2.2). The biochemical activity of the enzymes *in vitro* is

conserved as far as it has been queried, but the integration of the enzymes into the metabolic network and fitness of the diverse organisms is not. This latter point suggests we have much to learn about the selection for and the evolution of this protein superfamily. Implications on the chemistry of the metabolic network and the composition of the cellular milieu are discussed elsewhere (56).

2.8 Discovery of RidA biochemical mechanism challenged assumptions on reactions involving free water.

Elucidation of the RidA mechanism challenged our perspective on cellular water availability and required greater scrutiny be applied to our understanding of cellular and *in vitro* aqueous chemistry. The direct kinetic parameters of RidA are difficult to address due to the rapid turnover of 2AA in an aqueous environment. The inability to quantify 2AA concentration and the competing hydration with solvent water in a reaction mixture required indirect measurement of RidA kinetics through coupled reactions that measure the end product, pyruvate. In the coupled reaction, the 2AA generator, IlvA, forms 2AA and the addition of RidA increases the rate of pyruvate formation (9). With this experimental design the catalytic intermediate, assumed to be 2AA, must leave the enzyme active site to be hydrolyzed by RidA. These data 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 RidA enzyme to catalyze a reaction previously attributed solely to water has been demonstrated with several bacterial enzymes.

2.9 RidA extends beyond isoleucine biosynthesis

The RidA paradigm was first described using *S. enterica*, which established the connection of RidA to the isoleucine biosynthetic pathway (38-40, 44, 45, 57) (Figure 2.3). However, 2AA is generated as a catalytic intermediate by various PLP-dependent enzymes (46, 47, 49, 58-63). In *S. enterica*, 2AA is generated and released by threonine/serine dehydratase (IlvA, EC 4.3.1.19), cysteine desulfhydrase (CdsH, EC 4.4.1.15), and diaminopropionate ammonia-lyase (DapL, EC 4.3.1.15) (9, 64, 65) (Figure 2.3). To date, the PLP-dependent enzymes known to be inactivated by 2AA are fold type II, including isoleucine transaminase (IlvE, EC 2.6.1.42) (3, 38, 66), alanine racemase (Alr/DadX, EC 5.1.1.1) (67), serine hydroxymethyltransferase (GlyA, EC 2.1.2.1) (67), and aspartate aminotransferase (ApsC, EC 2.6.1.1) (52). In each case, the PLP-dependent enzyme target specific activity was decreased, not eliminated, in a *ridA* mutant under 2AA stress. The physiological relevance of RidA enzymes across domains appears to be conserved as far as it has been observed in eukaryotic organisms, *S. cerevisiae* and *A. thaliana* (50, 68-70). The PLP-enzymes described as 2AA-generators or 2AA-targets are found in all domains of life. Interestingly, RidA and threonine dehydratase are co-localized in *A. thaliana* and are co-expressed during development of maize and *A. thaliana* demonstrating the RidA enamine stress paradigm is likely conserved in eukaryotic organisms (50).

2.10 The RidA paradigm extends beyond *S. enterica*

The RidA paradigm was uncovered using *S. enterica* genetics and biochemistry. In the cell, free 2AA meets one of three fates i) diffusion throughout the cell and reaction with various PLP-dependent enzymes, possibly inactivating them, 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 as RidA increases

pyruvate formation, it simultaneously lowers the intracellular pool of reactive 2AA. The RidA paradigm demonstrated a novel biochemical function, usually attributed to cellular water. This finding indicates there are likely other reactions (discussed in section 2.14) predicted to be spontaneous that, are instead carried out by enzymes.

Rid enzymes from all domains of life complement a *S. enterica ridA* mutant. The complementation of *S. enterica ridA* mutant by RidA enzymes from diverse organisms supports the conserved deaminase function of RidA enzymes and suggests specific protein-protein interactions are not required to mediate activity. Accumulation of 2AA in a *S. enterica ridA* mutant is detrimental to growth and can be achieved by exploiting three known reactions that proceed through a 2AA intermediate followed by conversion to pyruvate. Growth defects in a *S. enterica ridA* mutant are elicited by growth on pyruvate through endogenous serine pools used by IlvA, exogenous serine that is used by IlvA in minimal glucose medium, exogenous cysteine used by CdsH, and/or exogenous diaminopropionate that is used by DapL (38, 39, 55, 64). Under these growth conditions in a *S. enterica ridA* mutant, RidA proteins from all domains expressed *in trans* complement the growth defects caused by 2AA accumulation. The conservation of 2AA deaminase activity *in vivo* supports the conserved biochemical function shown *in vitro*. However, the strong correlation between biochemical and biological activity of RidA enzymes does not assume the effects of 2AA damage nor the 2AA-enzyme generators (and substrates) are conserved throughout all organisms.

The sum of the data *in vitro* and *in vivo* resulted in a thorough understanding of the RidA paradigm using the *S. enterica* system as a model. Critically, the data from multiple approaches aligned to define and support this paradigm, that has been extrapolated to other systems and organisms. This paradigm defined key parameters that have been used to attribute effects to a

similar system, i.e., exacerbation by serine and/or cysteine and/or diaminopropionate, correction by isoleucine (via allosteric regulation of the generator enzyme, threonine/serine dehydratase) or other organism specific additions (via bypassing the perturbations to the networks). A conserved biochemical activity for RidA proteins has been demonstrated numerous times, with proteins from all domains of life and in several prokaryotes and eukaryotes, yet, the biochemical designation of RidA proteins is often lacking in data analysis present day. Without the correct annotation of RidA proteins and understanding of their conserved biochemical activity and established role in endogenous stress, meaningful data analysis may be lacking. This issue calls for correct and consistent gene annotation to move the understanding of RidA in complex organisms forward.

2.11 Metabolic integration of RidA influences phenotypic output.

With a solid understanding of the RidA paradigm in *S. enterica* and confirmed conservation of biochemical activity of enzymes from diverse organisms, integration of RidA into the metabolic networks of other organisms is of interest. Phenotypic analysis was a starting point to ask, what happens to diverse organisms when RidA is removed from the metabolic network? We found the RidA paradigm extended to other organisms, however, the metabolic architecture of each organism gave rise to phenotypic diversity. Despite the conservation of all enzymes relevant to the RidA paradigm in *E. coli* and *S. enterica,* removing RidA from the metabolic system had drastically different consequences in the closely related organisms (52). In an *S. enterica ridA* mutant, the primary 2AA-generator was the serine/threonine dehydratase (IlvA), the critical 2AA-target leading to a growth defect was the serine hydroxmethytransferase (Gly), and the branched-chain aminotransferase (IlvE) can be used as a proxy to observe 2AA damage. In *E. coli*, neither a growth defect, nor 2AA damage to IlvE is observed by the elimination of *ridA* alone or by elimination of *ridA* and other encoded Rid family members (*tdcF* and *rutC*). These data indicate the integration

of RidA into the metabolic networks of *S. enterica* and *E. coli,* and the way each organism elicits and responds to 2AA stress differ more than genetic comparison may suggest. These data were the first indication that the biochemical activity of RidA could be conserved, but the consequences to the organism different.

Pseudomonas aeruginosa encodes eight members of the Rid superfamily, two of which are annotated RidA (PA3123 and PA5339) (53). In *P. aeruginosa* only a mutation in PA5339 (*ridA*) led to metabolic defects. Similar to *S. enterica*, when *ridA* is absent in *P. aeruginosa*, there was a significant motility defect that was corrected by exogenous isoleucine (53, 71). The growth defect of the *P. aeruginosa ridA* mutant when exogenous serine was titrated in the growth medium was more severe than *S. enterica* or *E. coli ridA* mutants (Figure 2.4). The *P. aeruginosa ridA* mutant was sensitive to lower concentrations of serine and only slightly sensitive to cysteine compared to a *S. enterica ridA* mutant. *P. aeruginosa* encodes three generators of 2AA, two serine/threonine dehydratases (IlvA) and one cysteine desulfhydrase (CdsH). The increased sensitivity of *P. aeruginosa ridA* mutants to serine suggested 2AA accumulation proceeded largely through IlvA, but unlike *S. enterica ridA* mutants, only isoleucine restored metabolic defects in the *P. aeruginosa ridA* mutant despite encodings three glycine hydroxymethyltransferase homologs (GlyA), alanine racemases (Alr and DadX) and a putative aspartate aminotransferase. The differences of *S. enterica* and *P. aeruginosa ridA* mutant phenotypes further underscores the metabolic distinctions between organisms.

Campylobacter jejuni encodes two members of the Rid superfamily, a RidA and a Rid2 subfamily member (54). Similar to *P. aeruginosa* and *S. enterica ridA* mutants, *C. jejuni ridA* (*ridA*/*cj1388*) mutants have flagellar defects that indicate there may be far reaching effects of 2AA accumulation despite disparate metabolic networks. In undefined rich medium, the *ridA* mutant

exhibits reduced motility, decreased autoagglutination, phage infectivity, and visible flagellar defects (54, 72). However, *P. aeruginosa* and *S. enterica ridA* mutant motility defects rely on minimal medium where the addition of serine exacerbates the defect and isoleucine corrects it. Targeted genetics showed the biosynthetic serine/threonine dehydratase (IlvA) in *C. jejuni* was not responsible for 2AA accumulation and motility defects under the conditions tested. The phenotypes of the *C. jejuni ridA* mutant in undefined/rich media suggest damage to GlyA, IlvE, or AspC are not likely to cause the flagellar defects observed in *C. jejuni*. These data further emphasize how metabolic difference of unique organisms can impact our understanding of the role of RidA.

2.12 RidA plays an important role in eukaryotic organisms.

The function of RidA in *S. cerevisiae* highlights the essential role RidA protein may play in eukaryotic systems. *S. cerevisiae* encodes two RidA enzymes, a cytoplasmic and a mitochondrial-targeted RidA. The loss of mitochondrial RidA, Mmf1p (Mitochondrial matrix factor), leads to 2AA accumulation from serine dehydratases (Ilv1p or Cha1p, EC 4.3.1.19) in the mitochondrion, causing metabolic stress and irreversible loss of mitochondrial DNA (70). Mutants of the mitochondrial RidA, have reduced growth on dextrose and the addition of isoleucine or threonine (isoleucine precursor) restore growth (Figure 2.5). In the absence of the mitochondrial RidA, respiration on glycerol was abolished and, interestingly, addition of an iron chelator to the growth medium restored respiration capacity. Thus, in the context of the yeast mitochondrion, RidA appeared to influence iron homeostasis and further emphasize the critical and distinct role RidA plays in cellular function.

A. thaliana and maize each have a single RidA that is co-expressed with serine/threonine dehydratase and branched-chain aminotransferase-3 (BCAT3 or IlvE from *S. enterica*) from isoleucine biosynthesis plant during development (73). In defined growth medium without serine, the root growth of *A. thaliana ridA* mutants was significantly less than wild type plants and growth was further impeded by adding serine to growth medium. The root growth defect in *A. thaliana* was partially restored by addition of isoleucine, which may have indicated an isoleucine deficiency in the *ridA* mutant caused by 2AA damage to BCAT3 or, the more likely scenario, exogenous isoleucine allosterically inhibited the serine/threonine dehydratase 2AA production, thus, bypassing the need for RidA in *A. thaliana* as is the case in *S. enterica ridA* mutants (38, 74). The importance of RidA in *A. thaliana* root and plant development was further emphasized in a proteomic study of roots grown under exclusively ammonium or nitrate growth conditions. RidA (AT3G20390) was the most highly abundant protein under nitrate conditions. Although the authors of that study did not characterize the importance of RidA in plant roots, these studies underscore the importance of RidA enzymes in eukaryotic organisms and the need for additional genetic examination (75).

The impact of eliminating RidA from the metabolic network depends on the distinct metabolic architecture of the particular organism and results in diverse phenotypes. The phenotypic diversity suggests the integration of RidA in the metabolic network differs, it does not suggest various activities of the enzymes. However, diverse functions have been assigned and propagated throughout the literature for RidA enzymes across domains. It is essential that knowledge be assimilated across disciplines/fields as RidA enzymes across domains have been demonstrated *in vitro* and *in vivo*, in *S. enterica,* to share a conserved biochemical activity. Going forward, it is important that study of RidA enzymes include examination of the critical Arg105 (or

Arg 165) variants to demonstrate whether the conserved deaminase activity of RidA is required for the other activity in question. Similarly, it is key that *ridA* deletion mutants be generated to fully understand the physiological role of RidA enzymes in the metabolic context of the organism of study. With the long history of various assigned activities given to RidA enzymes, it is important to understand how the conserved deaminase activity influences these roles and if they are physiologically relevant.

2.13 The physiological role of Rid1-7 subfamilies remains unknown.

The Rid superfamily was defined using protein sequence homology from all domains of life (1). While RidA is found in all domains, Rid1-7 subfamily members are found only in prokaryotes and the number of subfamily members per organism is not consistent throughout bacterial species. For example, the *S. enterica* genome encodes RidA and YoaB and STM1549, which are assigned to subfamily Rid2 and Rid7, respectively. The genome of *E. coli* encodes five putative Rid proteins, *P. aeruginosa* encodes eight, and *C. jejuni* encodes two (52, 54, 72). Biochemical surveys and solved protein structures have begun to probe the roles of Rid1-3 proteins, whereas, 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. Our understanding of the Rid1-7 subfamilies is in the early stages and represents an area of interest moving forward. Many questions remain with regard to the function and physiological roles of the Rid1-7 subfamily members. Does function correlate with subfamily assignment? Do the subfamily members perform similar functions that are currently considered spontaneous? And will there be a phenotypic consequence of removing subfamily members or was the substrate of RidA uniquely reactive?

In addition to PLP-dependent dehydratases, some FAD- or NAD- dependent amino acid oxidase enzymes generate imine intermediates in their catalytic mechanisms. Table 2.3 shows validated Rid1-3 deaminases that acted on 2AA, generated by PLP-dependent dehydratases, as well as a broad range of imine intermediates, generated by FAD- or NAD- dependent amino acid oxidases, *in vitro* (1, 76). 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. Rid2 and Rid3 proteins deaminated iminoarginine, *in vitro*, and ultimately facilitated the overall rate of a pathway for D-arginine metabolism (76). While bacteria have few L-aminoacid oxidases (LOX), several of these enzymes are commercially available and thus have allowed assay of deaminase activity of RidA enzymes, despite the non-physiological context of these enzymes (1, 77). Due to the restrictions of RidA kinetic measurements, it is unclear whether RidA acts on the enamine or imine products of IlvA and CdsH, nevertheless RidA activity on imine substrates of LOX could suggest imines are the substrate of RidA.

The current evidence suggests that 2AA is unique in its ability to elicit an endogenous stress response. Compared to 2AA, 2AC has one additional methyl group, yet, it did not impede growth of a *ridA* mutant strain nor damage 2AA-susceptible enzymes *in vitro.* Rid enzymes deaminated both 2AC and 2AA, and both metabolites accumulated in a *ridA* mutant (9, 45). Given these observations, it is not appropriate to assume that all enamine/imine intermediates will lead to metabolic stress and observable phenotypes. Rid enzymes might deaminate enamine/imine intermediates in order to facilitate efficient metabolism in the cell where water availability is low (9, 78, 79).

2.14 Gene synteny may lead to functional understanding of Rid subfamily members.

Prokaryotes usually encode at least one RidA along with other members of the Rid subfamily. For example, *P aeruginosa* PA01 encodes eight bioinformatically identified Rid proteins including, four RidA (two with active site Arg105), one Rid1, and three Rid2 proteins (1, 80). Gene synteny may provide clues to Rid superfamily protein function and suggests that some Rid subfamily members may facilitate increased reaction rates and use substrates other than 2AA (1). PA5303, a Rid2 subfamily member, is regulated by agmatine; putrescine; and D/L-alanine within the operon of critical alanine catabolism genes, *dadX* and *dadA* (81, 82). The PLP-dependent enzyme, DadX (alanine racemase, EC 5.1.1.1) and DadA (D-amino acid dehydrogenase, EC 1.4.5.1) are responsible for the conversion of L-alanine to pyruvate and ammonia and it is conceivable that PA5303 increases the rate of this reaction (82, 83). Elimination of either *dadX* or *dadA* led to an inability for mutants to grow on L-alanine, however, to date, there has not been a phenotype associated with the loss of PA5303, nor has a biochemical role been determined *in vitro*. Additionally, PA5083 (Rid2) or *dguB*, is encoded in the *dguABC* operon that is positively regulated by exogenous D-Glu and D-Gln (84). DguA is a FAD-dependent dehydrogenase required for growth on D-Glu. While the role of PA5083 in D-Glu metabolism has not been explored, Rid2 enzymes (PA0814 and PA5083), each increase the rate of FAD-dependent reactions *in vitro* (76). Understanding the physiological importance of Rid1-3 subfamilies is an exciting avenue of research which may change our understanding of the mechanisms of certain metabolic reactions.

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 protein substrates, as was described for the FAD-dependent dehydrogenase DauA (76). Compelling gene clusters and/or biochemical evidence suggests Rid enzymes might function in pathways involved in antibiotic synthesis (85) or degradation of aromatic compounds (12, 13, 14 2003, 86, 87). These studies present exciting opportunities to examine the impact of non-2AA enamine/imine intermediates on bacterial metabolism.

2.15 A disparity between Rid1-3 and Rid4-7 subfamilies is addressed.

The overwhelming number of RidA structures can now be compared to a few Rid1 and Rid2 structure-function studies that incorporated soaking relevant ligands into active sites. These initial structural and biochemical studies have guided hypotheses that can be tested *in vivo*. Additional phenotypes will likely be uncovered by probing the relationship between FAD-enzymes and Rid enzymes *in vivo* in organisms such as *Pseudomonas* species that encode multiple Rid proteins. However, these phenotypes will likely be subtle as, unlike 2AA, metabolic intermediates may not be toxic.

Our understanding of Rid4-6 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.

2.16 Are Rid proteins multi-functional?

Over the past two decades, despite demonstration of the conserved biochemical activity of RidA enzymes, various additional activities have been assigned to RidA. The PLP-dependent enzyme damage caused by an accumulation of 2AA in a *S. enterica* mutant lacking *ridA* has been convincingly recapitulated *in vitro* and, in other organisms, phenotypes arise indicative of 2AA damage to enzymes. Every RidA protein expressed *in trans* has complemented the phenotype of a *S. enterica ridA* mutant. These data emphasize the critical role deaminase activity has in the cell. However, even after this paradigm was firmly established, several studies have reported other function/activity of RidA enzymes and ignored structural information about the critical Arg105 residue or the conserved deaminase activity. These reports raise the questions i) are the other observed effects and distinctive functions/activities traceable to the conserved deaminase activity, or, ii) are there additional biochemical activities that can be demonstrated to be relevant *in vivo*? In none of the cases described below have these two possibilities been definitively addressed.

The identification of a ubiquitous superfamily of unknown function spurred broad interest in biochemical characterization of the protein from the three domains of life. As a result, the collection of Rid superfamily knowledge includes localization studies in many cell types, data on expression patterns, and reports of protein modifications that might influence activity in the cell. Quite often, citations are limited to previous reports of the same activity, which limits exploration of the superfamily as a whole. In order to assimilate these currently disparate activities, it is critical to evaluate which are physiologically relevant. Current and emerging mutant phenotypes that arise in the absence of the critical (if there are multiple) RidA are expected to be sequelae of 2AA stress, and we present the biochemical activities that have been associated with RidA proteins in this light. Every RidA homolog tested to date catalyzes 2AA deamination and therefore, the only

biochemical activity that unifies proteins across domains. Therefore, it is critical to establish whether 2AA deamination is relevant to the mechanism of any additional function/activity in order to establish whether Rid proteins might serve moonlighting functions that are distinct from 2AA deamination. 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.

2.17 Localization of RidA proteins could indicate physiological importance.

High-throughput untargeted methods as well as targeted studies have identified RidA homologs in multiple types of eukaryotic cells. RidA (formerly perchloric acid soluble protein, PSP) was found in various eukaryotic tissues including the lung (19), digestive tract (21), liver (5-7, 10, 22, 27, 88, 89), kidney (5, 6, 20, 90), brain (89), white blood cells (6, 11), and oocytes (91). Changes in intracellular protein localization were correlated in rat kidney tissue treatment with various compounds intended to elicit endoplasmic reticulum stress (20). In plant cells, RidA was localized to the chloroplasts (with threonine dehydratase) (50, 92) and in yeast, RidA (Mmf1) was found in the mitochondria and in the cytoplasm (Hmf1) (68, 93). In bacteria, RidA proteins lack secretory tags and are predicted to remain in the cytosol (94), 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 (95). Taken together, these reports indicate localization to specific cellular organelles may suggest an essential role for RidA in these distinct cellular environments.

The broad complementation of *S. enterica ridA* mutant with diverse homologs from the three domains suggests that a conserved 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 high-throughput yeast two-hybrid using

Helicobacter pylori established that RidA (HP0944) interacted with and chromosomal replication initiator, DnaA/HP1529, the 50S large ribosomal subunit, HP1318, and other proteins unrelated to DNA/RNA under the conditions tested (96), though, the physiological relevance of these interactions was not confirmed. Immunoprecipitation studies suggested RidA proteins may interact with Y-box protein (YB-1) to regulate glucocorticoid receptor mediated mRNA degradation in vascular smooth muscle (11, 97). Protein-protein interactions of RidA enzymes should be demonstrated to be physiologically relevant to ensure interactions are not an artifact of experimental conditions.

In multiple scenarios, antibodies to a RidA homolog linked the enzyme to human health and may suggest antigenic properties. The human RidA enzyme UK114 has been associated with tumor growth, and UK114-specific antibodies appeared to have inhibitory properties, reducing metastatic masses (88, 98, 99). The connections between 2AA stress and cancer biology have been discussed elsewhere (100). Recent data suggests the RidA protein (Der f 34) found in the house dust mite (*Dermatophagoides farina*e) feces is a pan allergen associated with human dust mite and fungal allergies (51). Human development of antibodies to an allergen protein (RidA) in dust mite feces implies that RidA is secreted extracellularly or is exposed with other cytosolic contents after cell lysis.

If RidA enzymes are capable of 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 exploit the critical residues of the protein and test 2AA deaminase null variants to probe the connection to 2AA stress in this context.

2.18 Expression or abundance of RidA proteins is a field right for extrapolation.

Numerous studies found patterns in Rid expression that correlated with different cellular processes or metabolites in various organisms. These observations provide incentive for additional experimentation to test potential Rid connections. Most of the gene expression or protein abundance data represent 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 outside of the prokaryotes. Eukaryotic studies suggested RidA homolog expression could be linked to cell differentiation (6), healthy cell proliferation (101, 102), glucose fasting (103), meiosis in oocytes (91), *Giardia duodenalis* host attachment (104), and that expression might be influenced by promoter methylation in some cases (27).

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*. Other correlations have been drawn between Rid expression and growth at higher temperatures in *Clostridium difficile* (105), amino acid metabolism in *P. aeruginosa* (82) and *Streptococcus pyogenes* (106), or D/L-alanine metabolism in *P. aeruginosa* (81). A RidA homolog in *S. pyogenes*, was upregulated in strains with a defective two-component regulatory system (107). The effect of a *ridA* mutation on the transcriptional profile in a cell has been probed in bacteria and yeast (71, 108). A transcriptomics study using an *S. enterica ridA* mutant correctly predicted a defect in motility and demonstrated the wide-reaching affects a *ridA* mutation has on metabolism.

P. aeruginosa RidA proteins, PA5339 and PA3123 have been highlighted in numerous studies focused on adaptive stages of *P. aeruginosa*, yet, their importance in these studies has not been assessed. Interestingly, RidA (PA5339) accumulated cells in early attachment (ie., early

biofilm formation) compared to cells of unattached bacteria (109). However, in this study the *P. aeruginosa* RidA was misannotated and categorized with "RNA degradation" proteins, again emphasizing the need for consistency in annotation. In a study of the transcriptome of swarming *P. aeruginosa* colonies, PA3123 was down-regulated under swarming conditions, the protein was annotated as a "conserved" protein and was not the focus of the study (110). PA3123 was down-regulated 2-fold in swarm tendrils as compared to non-swarming colonies, during alginate-inducing conditions, and in the transition from planktonic growth to biofilm formation (111-113). PA3123 was shown to be up-regulated 2-fold under anaerobic urinary tract infection-type conditions (114). The inclusion of *P. aeruginosa* RidA proteins in several global data sets indicates there may be an important role for these proteins in changing environments that could influence invasion and virulence.

Unlike other bacterial RidA systems in *S. enterica*, *P. aeruginosa*, and *E. coli*, there is direct evidence that the *C. jejuni ridA* (*cj1388*) is regulated due to the changing metabolic needs of the cell and may be inversely correlated to iron availability (72, 115). Like *P. aeruginosa* RidA proteins, PA5339 and PA3123, *C. jejuni* RidA (strain specific gene designation 11168/*cj1388* or 81176/*cj1390*,) has been highlighted in numerous global -omics studies in *C. jejuni* (116-122). While *C. jejuni* RidA was not the focus of these studies, the frequency of RidA regulation in response to changing cellular conditions suggest *C. jejuni* RidA could play a direct or indirect role in virulence, antibiotic resistance, acid adaptation, growth with bile salts, and hydrogen peroxide and oxygen stress. When the data are taken together, these observations support the idea that RidA affects diverse metabolic processes in all domains of life.

RidA proteins from mammals were shown to have diverse functions and expression patterns, some of which can be explained in view of the RidA paradigm established in *S. enterica*

and others that cannot. RidA (P23) expression was enhanced in the liver (and kidney) of starved rats and after glucose was added to the diet *ridA* mRNA expression decreased (103). There is a plausible mechanism to account for enhanced RidA requirement under those conditions given the 2AA paradigm elucidated *in S. enterica*. Under starvation conditions, gluconeogenesis occurs in the liver where glucogenic amino acids are used to synthesize glucose. Serine, a precursor to 2AA from the serine/threonine dehydratase (IlvA) in *S. enterica*, is one of the best precursors to gluconeogenesis in the rat liver. PLP-dependent serine dehydratase (SDH EC 4.2.1.13) activity increases during glucose starvation and expression decreases during glucose feeding, following a pattern similar to *ridA* expression (123). Additionally, RidA (PSP1 from rat heart tissue) mRNA expression was upregulated on a diet supplemented with fatty acids which are also known to stimulate gluconeogenesis (124, 125). While pyruvate production for gluconeogenesis in the rat liver occurs in the cytoplasm, pyruvate is transported to the mitochondria for use conversation to oxaloacetate by pyruvate carboxylase, located in the mitochondrial matrix (126). Given the demonstration that mitochondrial RidA (Mmf1) is required for mitochondrial maintenance in the yeast, and gluconeogenesis requires mitochondria pyruvate carboxylase, it is also possible to conceive that mitochondrial protection via stress mechanisms during starvation may be enacted during mouse starvation and account for the upregulation of RidA (70). Use of mammalian *ridA* knockouts may begin to uncover a physiological relevance of the enzyme in multicellular organisms.

2.19 RidA enzymes as translation inhibitors

Eukaryotic RidA proteins were originally designated perchloric acid-soluble proteins (PSP) with endoribonuclease activity. Crude extracts or purification fractions containing RidA (formerly PSP) proteins from rat liver (5, 10) and human monocytes (6) inhibited translation in

cell-free assays. Heat treatment or precipitation via RidA-specific antibodies eliminated the effect on translation, suggesting RidA was responsible for the activity. Human RidA (called p14.5) was upregulated in monocyte/macrophage differentiation (6). The p14.5 protein was shown to inhibit protein synthesis from Tobacco Mosaic Virus (TMV) mRNA with 2.5-20 μM range of purified protein (6). Similarly, rat liver RidA inhibited TMV mRNA translation in a dose dependent manner with 50% inhibition at 8 nM RidA (5). In these studies, RidA behaved differently than RNase A, required prolonged incubation, and caused dissaggregation of polyribosomes, suggesting RidA inhibited the initial stage of protein synthesis (5). Translation inhibition by rat liver RidA was further examined using mRNA encoding *E. coli* dihydrofolate reductase and endogenous globin mRNA (10). This study found RidA interacted with the mRNA, rather than translation machinery, suggesting RidA fragmented polysomal mRNA through ribonuclease activity. *In vitro*, RidA cleaved only single stranded regions of RNA, did not cleave tRNA or rRNA, and lacked histidine residue of other ribonucleases.

RidA enzymes have also been linked to mRNA degradation through associates with degradation proteins in monocytes (11) and *Entamoeba histolytica*, a protozoan parasite. A combination of the glucocorticoid, dexamethasone, Y-box affinity protein (YB-1), glucocorticoid receptor (GR) and RidA (UK 114) were shown to degrade monocyte chemoattractant protein 1 (MCP-1) mRNA (11). The MCP-1 mRNA degradation "complex" required dexamethasone modification/activation of one or more components prior to mRNA degradation, however, the data suggested no interaction between RidA and the MCP-1 mRNA, whereas, other components, GR or YB-1, produced gel shifts when incubated with MCP-1 mRNA. The role of RidA and glucocorticoid receptor mediated degradation of mRNA was further studied using Arg105 mutants of the protein, however, authors did not reflect on the conserved deaminase activity of the protein

nor structural studies that elucidated the necessity for the Arg105 residue in that activity (97). RidA (EhL-PSP) was associated with RNA degradation through direct or indirect associations with the deadenylase, CAF1, and exosome component, EhRRP41, in *Entamoeba histolytica.* RidA localized in the cytoplasm with CAF1 and in plasmatic membrane of *E. histolytica,* as well as coimmunoprecipitating with EhRRP41 despite only 20% co-localization in the cytoplasm, suggesting a possible role for RidA in mRNA degradation due to co-localization with degradation machinery. The endoribonuclease activity of RidA enzymes (from several different organisms) has been studied extensively, yet the conserved deaminase activity has not been considered in these studies. Similarly, a physiological requirement for the translation inhibition activity has not been demonstrated using knockout organisms or, rarely, with Arg105 mutants.

Interestingly, RidA (called Rhp-PSP) from *Rhodopseudomonas palustris* was shown to have antiviral properties against tobacco mosaic virus (TMV) (127). The purified protein was sprayed on tobacco plant leaves infected with TMV and a concentration-dependent inactivation of TMV was observed. Yet, the mechanism for purified RidA action again TMV was not investigated further. As discussed above, multiple studies that demonstrated the translation inhibitory effects of RidA on TMV mRNA (5, 6, 17). The studies taken together suggest the antiviral activity of RidA against TMV may be through interaction with mRNA. Perhaps RidA plays a role in bacterial immunity, presenting an attractive area 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 could potentially co-precipitate, resulting in the translation inhibition. This might explain discrepancies between native and recombinant proteins in some studies. 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 (Arg105 in *S. enterica* RidA) might simultaneously resolve whether an active RidA deaminase is required and could confirm whether RidA is responsible for the inhibitory effect on translation.

2.20 The role of RidA as a chaperone (or heat shock) protein is explored.

Early sequence alignments suggested areas of certain RidA subfamily proteins were homologous to calpain-activating proteins (15) or heat-shock proteins (4, 7), which prompted assessments of chaperone activity. Importantly, the confirmed activities were protein specific. Human RidA (UK114) purification fractions that catalyzed calpain activation contained additional cellular components (15), and chaperone activity was not detected with purified *Drosophila melanogaster* RidA (DUK114) (18). In contrast, DUK114 appeared to act as chaperone in *in vitro* citrate synthase assays and overexpression seemed to protect *Drosophila* embryonic cells from heat-shock (18). Recently, *Capra hircus* (Goat) RidA, UK114, was shown to be extremely heat resistant and to hydrolyze imino acids produced by L- or D-amino acid oxidases using L-leucine, L-alanine, L-methionine, or L-glutamine or D-leucine and D-alanine, respectively, which coincides with the substrates for *S. enterica* RidA (8). The sequence alignments for RidA and heat-shock proteins were not provided in references describing their similarity, however, human (p14.5) and mouse (Hrp12) RidA proteins have ~24% and *S. enterica* RidA has ~15% sequence identity to a portion of human heat-shock proteins, Hsp90AA1 and Hsp90AB1, but do not share sequence homology to DnaK from *E. coli.*

Based on the mutant phenotype consistency with 2AA stress observed for bacteria, plants, and yeast mutants that lack the relevant RidA homolog, any phenotypes that might arise independent of 2AA are exciting and may help to move this field of research forward. *E. coli ridA* mutants appear to be more sensitive to peroxynitrite or hypochlorite (HOCl) compared to wild type (128, 129). However, 2AA indirectly damages multiple nodes of the metabolic network (67, 130) 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 caused by peroxynitrite or HOCl ultimately stem from an inactivated PLP-dependent enzyme, and perhaps a new 2AA-susceptible enzyme might be identified. We propose a series of experiments that will help to establish whether emerging phenotypes ultimately stem from 2AA stress. First, establish the 2AA generator and target enzymes in a *ridA* mutant using the RidA paradigm that was established in *S. enterica.* If 2AA accumulation proceeds through serine/threonine dehydratase (IlvA) and if IlvA has an allosteric regulatory domain, phenotypes elicited by 2AA stress will be improved by supplementation with isoleucine, a nutrient that halts 2AA generation by allosterically inhibiting IlvA. If the critical target of 2AA damage in a *ridA* mutant is serine hydroxmethytransferase (GlyA), supplementation with glycine should circumvent 2AA-damage. To further screen for 2AA damage to known enzyme targets, the specific activities of 2AA-sensitive enzymes (threonine/serine dehydratase, alanine racemase, serine hydroxymethyltransferase and aspartate aminotransferase) can be tested and screening additional PLP-enzymes in fold-type I, III, and IV families might uncover a novel 2AA target. Decreased activity of any combination of these enzymes suggests the phenotypic 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).

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 seminal study by Muller, *et. al.* has been cited in multiple review articles (131, 132). Here, we address experimental concerns and suggest experiments to elucidate the RidA big picture. The sensitivity of a *ridA* strain to HOCl was evaluated in rich medium using a popular protein purification strain (128), which was inconsistent with a standard genetic approach. 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.

We provide an alternative interpretation of the data and propose experiments to distinguish between the hypotheses. *In vitro*, HOCl-modified RidA ($RidA^{HOCl}$) inhibited the rate of 2-ketobutyrate formation from IlvA with threonine (129). In addition to the interpretation that $RidA^{HOCl}$ and IlvA directly interact, other scenarios are possible and have not been addressed. If the HOCl modification is unstable and remains reactive, interaction with IlvA or the substrate might explain the loss of activity. $RidA^{HOCl}$ might be isolated from wild type *E. coli* following exposure to HOCl and could provide insight on the stability of the modification. RidA binds substrates with a carboxyl group, so it is also possible that binding threonine might prevent its dehydration by IlvA.

It is unlikely that $RidA^{HOCl}$ would universally interact with all *E. coli* 2AA generators, and interactions would not be expected with non-bacterial enzymes, so the effect of $RidA^{HOCl}$ on CdsH or *A. thaliana* IlvA activity might provide alternative platforms to evaluate activity of the modified protein *in vitro*. We propose that Arg105 might be targeted by HOCl and could account for the decreased deaminase activity. This is consistent with the observation that cysteine-free RidA

appeared to be chaperone-activated and lacked deaminase activity, and the prediction that arginine and lysine residues are most likely to be modified. Specific proteins (citrate synthase and IlvA) were assessed for *in vitro* aggregation (129), which might be addressed using an approach designed to assess whole-cell aggregation in a *ridA* strain compared to wild type. This might also identify the relevant proteins from the candidates that interacted with HOCl-modified RidA *in vitro*.

It is possible RidA might play a secondary role as a chaperone in addition to its primary function as a 2AA deaminase. 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 corresponded with a loss of deaminase activity, which suggests that the cell would meet increasing 2AA burdens if the RidA pool were converted to a chaperone *in vivo*. Loss of deaminase activity corresponds to 2AA accumulation and would lead to the decreased activity of several target PLP enzymes that ultimately impact metabolism. Therefore, it is expected that a *ridA* mutant strain growing in the presence of HOCl would experience 2AA damage as chaperone activity is activated.

2.21 RidA proteins are modified under different conditions.

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 (133, 134). In human liver cells, the genetic loci of the RidA homolog, p14.5, had DNA methylation patterns associated with aberrant proliferation, which was reminiscent of the mechanism of silencing tumor suppressing genes (108). *In vitro*, the mouse liver homolog was phosphorylated by a phosphate kinase (7). The purified rat heart RidA, PSP1, bound fatty acids in a 1:1 ratio (17). A lipoprotein probe extracted a bacterial RidA from *E. coli* lysate and suggested that the Cys107 residue might be capable of binding fatty acids (135). This residue was also modified following peroxynitrite

exposure (128). 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 the conserved deaminase 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 (128). This discrepancy was surprising, and the authors proposed the variant was constitutively active yet "redox-dead".

As 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 (128). Complementation data would bolster support for the conclusion that *ridA* is the relevant mutation. If an allele encoding RidA^{C107S} restores growth when expressed *in trans*, then these experiments would support the hypothesis that the variant protein is insensitive to peroxynitrite. We recommend these experiments be incorporated in future work but acknowledge that the published data are consistent with the prediction that deamination is unaffected by modifications to Cys107. As it is possible that RidA performs various functions in the cell in addition to its role as a 2AA deaminase, future studies should continue to investigate the delineation of these two functions and establish the physiological conditions in which each is relevant. Given the differences between the RidA metabolic networks of even closely related organisms such as *S. enterica, E. coli, P. aeruginosa,* and *C. jejuni* (52-54), exploring redox phenotypes in other organisms will likely uncover new metabolic connections.

Given the diversity of activities assigned to RidA proteins, it is important to understand if all activities can be recapitulated *in vitro* and *in vivo* to determine the physiological relevance. Additionally, given the far-reaching effects of 2AA damage *in vivo* and how those effect change

due to the unique metabolic architecture of each organism, it is important to understand if the other functions that have been observed for RidA enzymes, beyond the conserved deaminase activity, are due to direct or indirect effects.

2.22 Assimilation of Rid knowledge across fields

In light of extensive literature pertaining to RidA protein across domains of life, it is critical to reevaluate the existing knowledgebase. With "reference to sequence" online tools, such as paperBLAST, it is easier to identify studies addressing RidA biology using diverse model organisms spanning the domains of life. It is clear that RidA proteins play an important metabolic role in prokaryotic and eukaryotic metabolism and those roles may not be vastly different. RidA deaminase/deiminase activity is conserved across all domains of life and has been demonstrated with every RidA protein tested to date. All RidA proteins tested can complement in a *S. enterica ridA* mutant *in vivo*, suggesting this conserved biochemical function is ancient, essential, and requires no specific protein-protein interactions. RidA proteins from various eukaryotic systems have been studied for the last thirty years, often, without consideration of the conserved activity demonstrated for these proteins, nor the critical amino acid residues required for that activity. Moving forward, the field must consider the biochemical activity prescribed to all RidA proteins in order to create a working model of the protein function in the metabolic system of each organism.

2.23 RidA plays an important role beyond prokaryotes.

The extensive study of bacterial RidA is frequently under-cited in studies of eukaryotic RidA and has often been relegated to a role in isoleucine biosynthesis, which ignores the broader implications of 2AA stress and deaminase activity in all organisms (Figure 2.2). Bacteria provided a genetically tractable model system to understand the role of 2AA through a combination of

rigorous genetics and biochemical experiments. Similar experiments have been replicated in plants and yeast and support the idea that 2AA stress arises in the absence of a functional RidA in each of these cases. 2AA deaminase activity is the single activity conserved in every RidA protein isolated to date and has been confirmed using enzymes from all domains and in several independent experiments. Considering the 2AA target serine hydroxymethyltransferase is an essential enzyme in all life forms and serine/threonine dehydratase is a critical enzyme in gluconeogenesis, it is unlikely these effects are limited to bacteria. Together, these studies urge the investigation of the effects of this reactive molecule in all domains of life. We are missing broader understanding of the role of RidA enzymes without knockout organisms like mice, rats, zebrafish, fruit flies and more.

2.24 An unbiased approach in a model organism led to functional characterization.

A serendipitous mutation was identified within the context of thiamine biosynthesis that eventually led to elucidation of the RidA stress paradigm using *S. enterica* as a model system. It is likely that solely structure-function approaches would not have been successful at identifying 2AA as the relevant substrate, given its short half-life (~1s). Furthermore, development of relevant activity assays was possible only after the identification of PRA accumulation in a *ridA* mutant of *S. enterica*, a phenomenon unrelated to the enzyme deaminase 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 the beginning. Instead, simple models were built and refined, providing a strong case for the continued support of curiosity-driven research in the "-omics" age to unravel deeper metabolic connections.

2.25 Caveats of annotating a protein superfamily of unknown function.

RidA protein sequence analysis led ORFs to be clustered and classified prior to biochemical (biological) analysis. This strategy can have value in many aspects of exploratory research but can also result in lingering issues when annotation conceals biochemical and mechanistic information. Proteins are annotated from diverse organisms, using different experimental approaches and this can generate confusion in the literature. The Rid family is an example of the pitfalls created by this approach. 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, 136). Therefore, the Rid superfamily illustrates a quandary about functional analyses that resonates throughout the biological sciences. The final question is whether the all of the described functions are 1) extrapolatable to other family members, and 2) physiologically relevant in diverse organisms. The abundance of literature examining RidA enzymes; knowledge of its role in bacterial, plant, and eukaryotic stress; and indications of its role in mammalian physiology, allergies, and cancer emphasizes the need for all fields to fully recognize the history of research into this enzyme and consider the part that 2AA and/or deaminase activity may play in eukaryotic systems. RidA is highly expressed in the liver which is a critical organ for amino acid metabolism, RidA from goat liver acts as an imine deaminase *in vitro*, and human RidA (along with all other RidA proteins tested) deaminates 2AA *in vivo* (3, 5, 7, 8). To understand the role of RidA proteins in mammalian systems, knock-out organisms must be generated and tested in light of the RidA paradigm identified in *S. enterica* and other single-celled systems.

2.26 References

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Table 2.1 Available Rid structures

Rid subfamily	Protein	Gene name/locus tag	Organism	PDB	Ref	Deposit
RidA	PSPTO-PSP	PSPTO_0072	*Psuedomonas sryingae*	3K0T	(33)	2009
	RidA/YjgF	*ridA/yjgF*	*Escherichia coli*	1QU9	(23)	1999
	YabJ	*yabJ*	*Bacillus subtilis*	1QD9	(24)	1999
	Hmf1	YER057c	*Saccharomyces cerevisiae*	1JD1	(31)	2001
	L-PSP	Hrsp12	*Rattus norvegicus* (rat)	1QAH	N/A	1999
	UK114	Hrsp12	*Capra hircus* (goat)	1NQ3	(30)	2003
	Hp14.5	HRSP12	*Homo sapiens* (human)	1ONI	(26)	2003
	TdcF	*tdcF*	*Escherichia coli*	2UYJ	(29)	2007
	TdcF	*tdcF*	*Escherichia coli*	2UYP	(29)	2007
	TdcF	*tdcF*	*Escherichia coli*	2UYK	(29)	2007
	TdcF	*tdcF*	*Escherichia coli*	2UYN	(29)	2007
	ST0811	STK_08110	*Sulfolobus tokodaii*	1X25	(137)	2005
	HI0719	*HI_0719*	*Haemophilus influenzae*	1J7H	(2)	2001
	RidA	RIDA	*Arabidopsis thaliana*	5HP7	(36)	2016
	RidA	RIDA	*Arabidopsis thaliana*	5HP8	(36)	2016
	SPy2060	Spy_2060	*Streptococcus pyogenes*	2EWC	N/A	2005
	Cth_2968	Cthe_2798	*Clostridium thermocellum*	1XRG	N/A	2004
	PA3499	PA3499	*Pseudomonas aeruginosa*	2IG8	N/A	2006
	TTHA0137	TTHA0137	*Thermus thermophilus*	2CVL	N/A	2005
	TTHA0137	TTHA0137	*Thermus thermophilus*	2CW4	N/A	2005
	APE1501	APE1501	*Aeropyrum pernix*	2CWJ	N/A	2005
	Mmf1	Mmf1	*Saccharomyces cerevisiae*	3QUW	(34)	2011
	TM0215	TM0215	*Thermotoga maritima*	2B33	N/A	2005
	PH0854	PH0854	*Pyrococcus horikoshii*	2DYY	N/A	2006
Rid2	YoaB	*yoaB*	*Salmonella typhimurium*	3GTZ	N/A	2009
	NMB1025	NMB1025	*Neisseria meningitidis*	3KJJ	N/A	2009
	NMB1025	NMB1025	*Neisseria meningitidis*	3KJK	N/A	2009
Rid6	Rv2704	MT2777.1	*Mycobacterium tuberculosis*	3I7T	(32)	2009
Rid7	YjgH	*yjgH*	*Escherichia coli*	1PF5	N/A	2003
RutC	RutC	*rutC*	*Escherichia coli*	3V4D	(35)	2011

Adapted from the dissertation of Hodge-Hansen, 2018

Table 2.2 Distribution of Rid proteins in representative organisms

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

[a] Genomes and annotations accessed from the PubSEED database (138).

[b] Organisms additionally encodes RutC, a member of the Rid superfamily that has not been assigned a subfamily. *Adapted from the dissertation of Hodge-Hansen, 2018*

Table 2.3 Validated Rid deaminases

[a] RutC has not been assigned to a subfamily.

Adapted from the dissertation of Hodge-Hansen, 2018

Organism	Protein	Subfamily	Reference
Archaea			
Pyrococcus furiosus	PF0668	RidA	(3)
Bacteria			
Salmonella enterica	RidA	RidA	(9)
Salmonella enterica	YoaB	Rid2	(1)
Escherichia coli	RidA	RidA	(52)
Escherichia coli	TdcF	RidA	(52)
Escherichia coli	RutC	N/A[a]	(9)
Bacillus subtilis	YabJ	RidA	(9)
Acinetobacter baylyi	ACIAD3089	Rid1	(76)
Campylobacter jejuni	Cj1388	RidA	(54)
Campylobacter jejuni	Cj0327	Rid2	(54)
Pseudomonas aeruginosa	PA5339	RidA	(53)
Pseudomonas aeruginosa	PA0814	Rid1	(76)
Pseudomonas aeruginosa	PA5083	Rid2	(76)
Pseudomonas syringae	PSPTO_0102	Rid2	(76)
Pseudomonas syringae	PSPTO_3006	Rid3	(76)
Pseudomonas fluorescens	PFL_1385	Rid3	(76)
Yersinia pestis	Y3551	RidA	[Martínez, *in preparation*]
Eukarya			
Homo sapiens	UK114	RidA	(3)
Cucumis sativus	ChrD	RidA	(3)
Arabidopsis thaliana	RidA	RidA	(36, 50)
Zea mays	RidA	RidA	(50)
Dermatophagoides farinae (house dust mite)	Derf34	RidA	(51)
Capra hircus (goat)	UK114	RidA	(8)
Saccharomyces cerevisiae	Mmf1	RidA	(55)
Saccharomyces cerevisiae	Hmf1	RidA	(55)

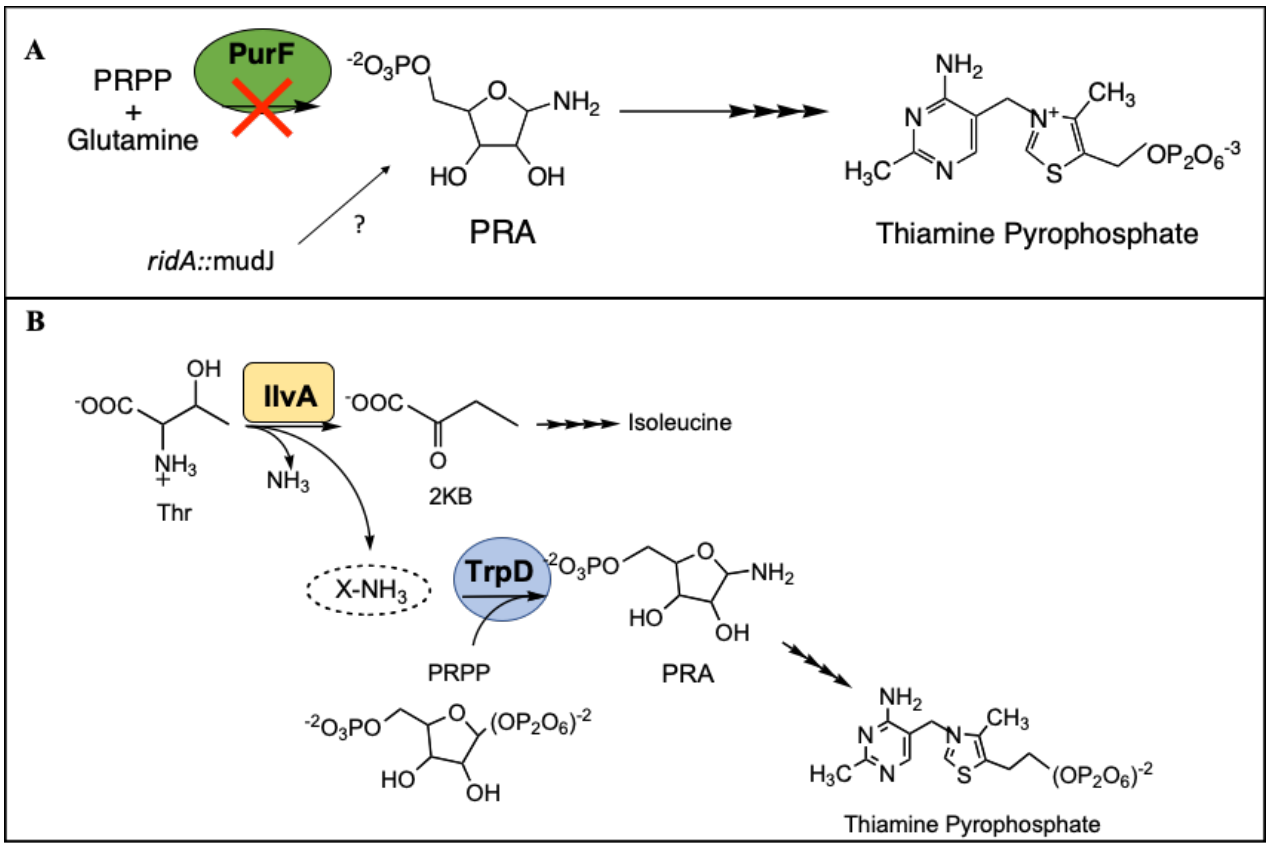

Figure 2.1. A null mutation in *ridA* allowed *S. enterica purF gnd* mutants to synthesize PRA. (A) Initial studies in a *S. enterica purF gnd* mutant identified a *ridA* null mutation that was able to synthesize PRA (phosphoribosyl amine) in a to overcome a thiamine auxotrophy. (B) The mechanism of PRA formation in a *purF gnd ridA* mutant required PRPP (phosphoribosylpyrophosphate), threonine, IlvA, TrpD, and an uncharacterized intermediate (X-NH_3). (40, 44, 45)

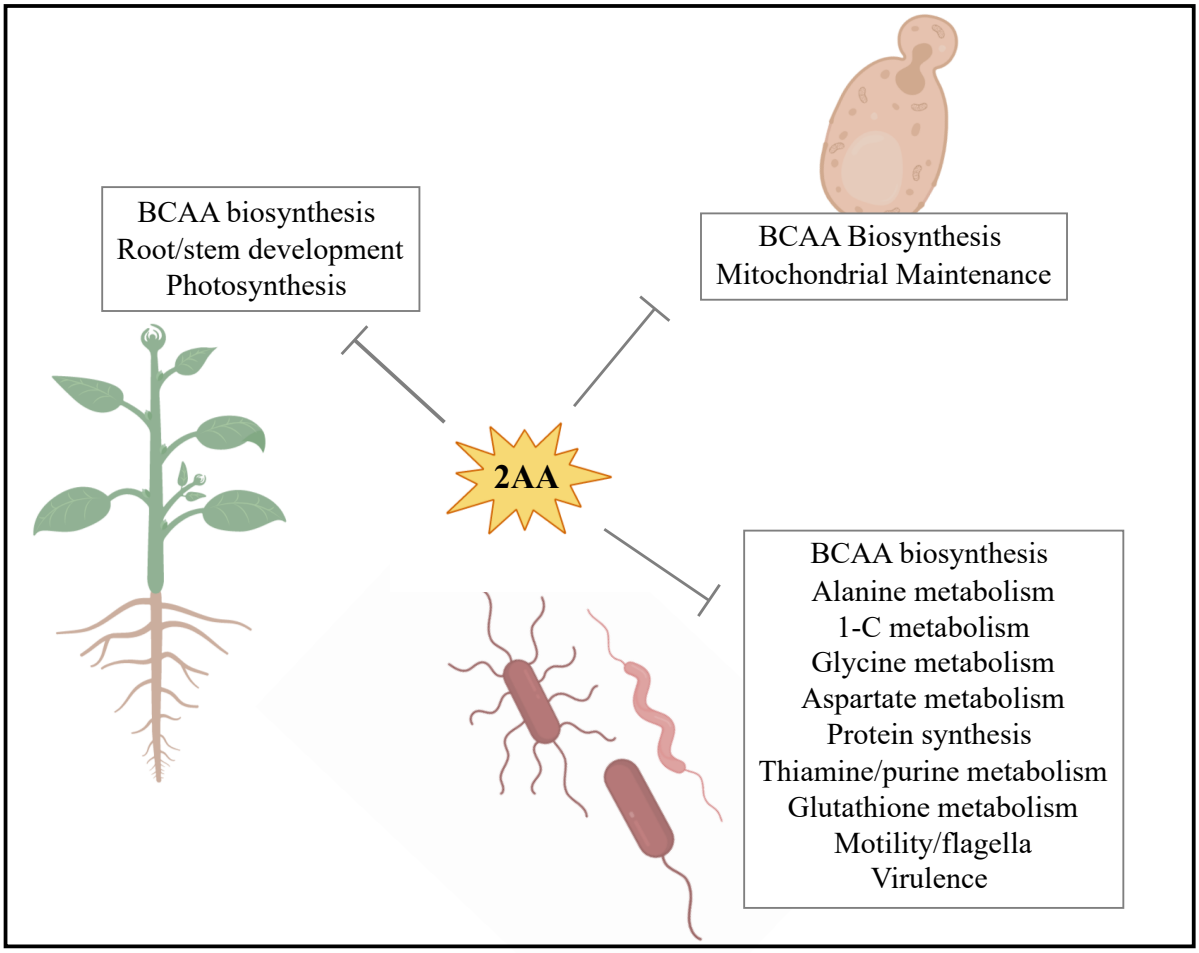

Figure 2.2 Mutant phenotypes of prokaryotic and eukaryotic organisms indicate pathways affected by lack of RidA. Phenotypes have been reported for mutant organisms that lack RidA from bacteria (*S. enterica* (38-40, 44, 57, 64, 67, 71, 130, 139), *E. coli* (52), *P. aeruginosa* (53) and *C. jejuni* (54, 72), plants (*A. thaliana* (1) and *L. esculentum* (92), and the yeast *S. cerevisiae* (68, 70, 140). This model assumes 2-aminoacrylate (2AA) damage directly or indirectly perturbs the metabolic network to affect these areas of metabolism. (*Figure created with Biorender.com*)

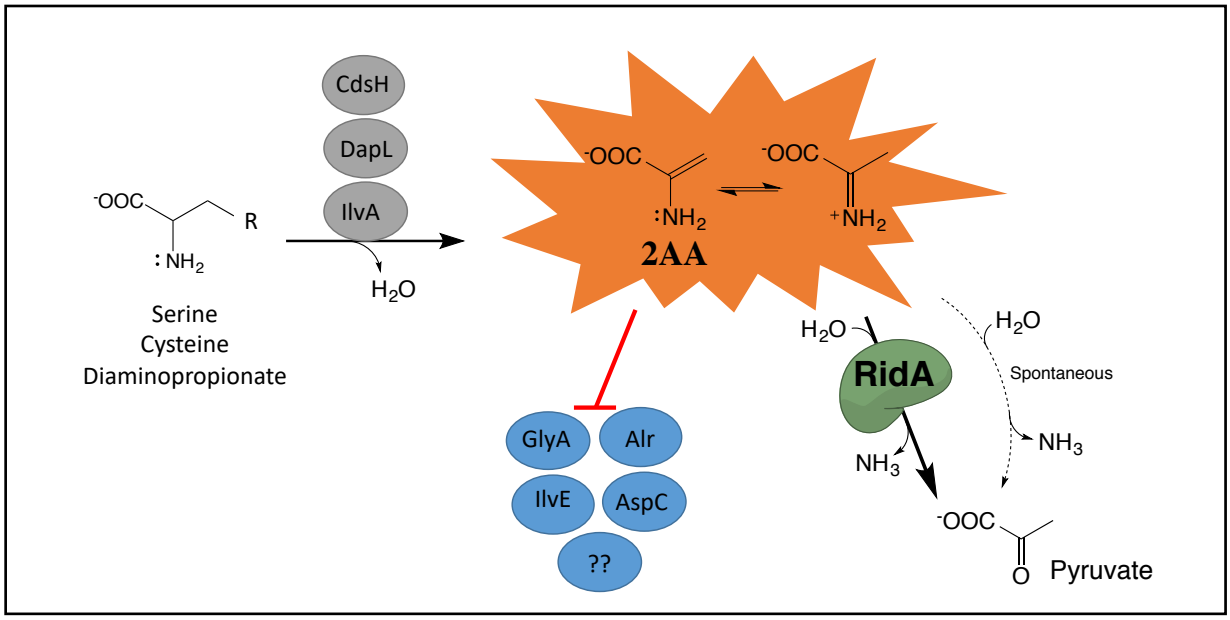

Figure 2.3 The RidA paradigm. The activities of some PLP-dependent enzymes (fold-type II, in grey) can lead to accumulation of 2-aminoacrylate (2AA, in orange). Free 2AA meets one of three fates: i) diffusion throughout the cell and inactivation of certain PLP-dependent enzymes (fold-types I and IV, in blue), ii) deamination by RidA (in green) or iii) non-enzymatic hydrolysis by solvent water. Inactivation of PLP-dependent enzymes through mechanism (i) can lead to metabolic defects while mechanisms (ii) and (iii) result in formation of the less reactive ketoacid.

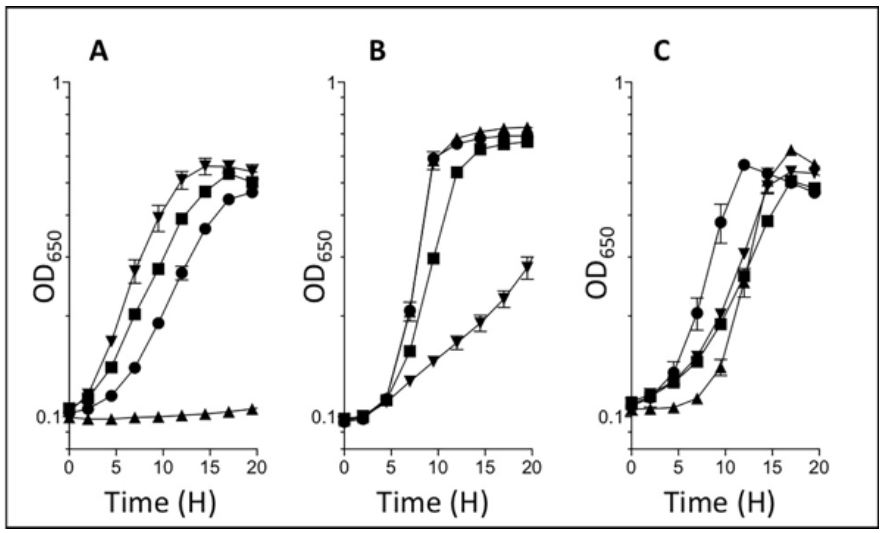

Figure 2.4: Sensitivity to serine differs among organisms that lack RidA. In minimal media with glycerol the addition of 3 mM serine caused varying growth defects in each organism. These data show 3 mM serine completely inhibits growth of a *P. aeruginosa* $_{pa}$*ridA* mutant (A), partially inhibits growth of the *S. enterica ridA* mutant, (B) and does not inhibit growth of the *E. coli ilvA219* Δ*ridA* Δ*tdcF* mutant (C).

(A) *P. aeruginosa* $_{pa}$*ridA* mutants grown in glycerol alone expressing, pEmpty vector (circle) or p $_{pa}$*ridA* (squares) or grown in glycerol/serine, pEmpty vector (triangles) or p $_{pa}$*ridA* (inverted triangles). (B) *S. enterica* grown in glycerol, wildtype (circle) or *ridA* mutant (squares) and in glycerol/serine, wildtype (triangles) or *ridA* mutant (inverted triangle). (C) *E. coli* grown in glycerol, *ilvA219* (represents wildtype growth, circles) or *ilvA219* Δ*ridA* Δ*tdcF* (squares) and in glycerol/serine, *ilvA219* (triangle) or *ilvA219* Δ*ridA* Δ*tdcF*
(inverted triangles). (53)

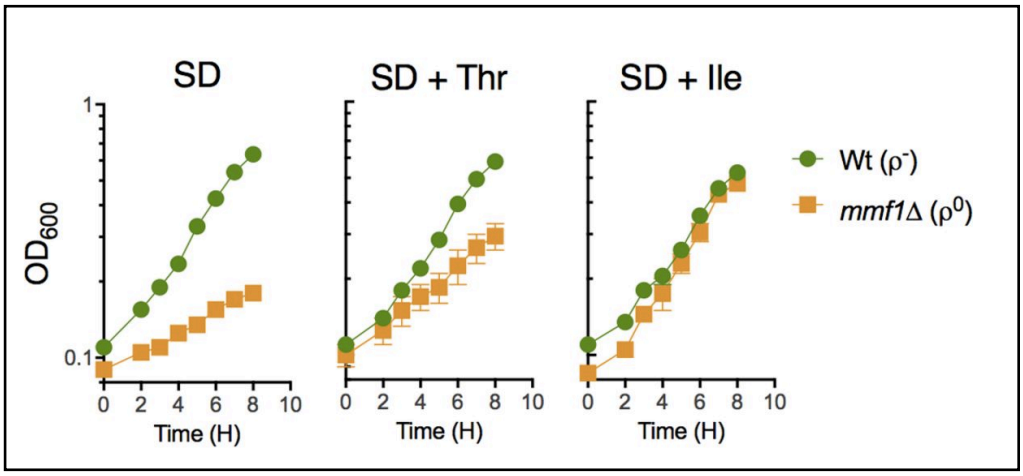

Figure 2.5: Yeast lacking the mitochondrial RidA (Mmf1p) have a growth defect that is improved with isoleucine or threonine. Growth of *p*° Δ*mmf1* mutant (orange square) or petite wild-type (*p*$^{-}$) strains in liquid synthetic dextrose medium supplemented with isoleucine or threonine as indicated above graph. Isoleucine, and to a lesser extent, threonine improve growth of the mitochondrial *ridA* (*mmf1*) mutant, suggesting 2AA is generated by serine/threonine dehydratases in *S. cerevisiae*. (70)

CHAPTER 3

Cj1388 IS A RIDA HOMOLOG AND IS REQUIRED FOR FLAGELLA BIOSYNTHESIS AND/OR FUNCTION IN *CAMPYLOBACTER JEJUNI*[1]

[1]Irons JL, [2]Sacher JC, [2]Szymanski CM, and [1]Downs DM. 2018. Frontiers in Microbiology.10: 2058. [1]Experiments were designed, carried out, and data analyzed by Irons JL. [1]Manuscript was drafted by Irons JL and Downs DM.

3.1 Abstract

Campylobacter jejuni is the leading bacterial cause of acute gastroenteritis worldwide and thus significant to public health. *C. jejuni* primarily lives in the gastrointestinal tracts of poultry and can contaminate meat during processing. Despite a small genome, the metabolic plasticity of *C. jejuni* allows proliferation in chicken ceca and mammalian host intestines, and survival in environments with a variety of temperatures, pH, osmotic conditions, and nutrient availabilities. The exact mechanism of *C. jejuni* infection is unknown, however, virulence requires motility. Our data suggest the *C. jejuni* RidA homologue, Cj1388, plays a role in flagellar biosynthesis, regulation, structure, and/or function and, as such is expected to influence virulence of the organism. Mutants lacking *cj1388* have defects in motility, autoagglutination, and phage infectivity under the conditions tested. Comparison to the RidA paradigm from *Salmonella enterica* indicates the phenotypes of the *C. jejuni cj1388* mutant are likely due to the inhibition of one or more pyridoxal 5'-phosphate-dependent enzymes by the reactive enamine 2-aminoacrylate.

3.2 Introduction

The Rid/YER057c/UK114 protein superfamily (COG0251) is broadly conserved throughout all domains of life (1-7). Based on phylogenetic analysis, the superfamily was divided into eight subfamilies; RidA, which includes homologs of the archetypical protein from *S. enterica*, and Rid1-7, which are not well understood (5). Prokaryotic genomes can encode several members of the Rid1-7 subfamilies while also encoding one or more RidA proteins. In many genomes, the RidA homologs are not annotated with the ascribed biochemical function for these proteins. The RidA, reactive intermediate deaminase A, of *Salmonella enterica* was found to be an enamine deaminase, and multiple homologs from the three domains of life have similar activity (3, 5, 6, 8, 9). In some organisms, a cellular role for RidA involves quenching the reactive

metabolite 2-aminoacrylate (2AA) to prevent damage to specific pyridoxal 5'-phosphate (PLP)-dependent enzymes (4, 5, 10-16). RidA homologs have a similar role in at least *Escherichia coli*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae,* although the phenotypic consequences of a *ridA* mutation depends on the specific metabolic network architecture of the organism (9, 16, 17). Enzyme damage resulting from accumulated 2AA can impact growth, motility, biofilm formation, iron homeostasis and, potentially virulence.

Campylobacter jejuni NCTC 11168, a prominent diarrheal pathogen, encodes two members of the Rid superfamily, a RidA homolog (Cj1388*)*, and a protein from the Rid2 subfamily (Cj0327). Data presented herein confirmed Cj1388 is a RidA protein and for clarity this locus is designated *$_{cj}$ridA* throughout. Previous data suggested that *C. jejuni* Cj1388 ($_{Cj}$RidA) plays a role in flagella-flagella interactions, possibly through regulation of flagella glycan modification (18). Additionally, $_{Cj}$RidA has been highlighted in several global -omics studies in *C. jejuni* strains 11168 and 81176 (strain specific gene designation *cj1388* or *cj1390*, respectively) (19-25). These data sets suggest $_{Cj}$RidA could play a direct or indirect role in virulence, antibiotic resistance, acid adaptation, growth with bile salts, and hydrogen peroxide and oxygen stress. Finally, *$_{cj}$ridA* is a member of the HeuR (Heme utilization regulator) regulon (18, 26). HeuR is a PAS-domain containing regulator, and thought to be regulated in response to changing environmental cues (18, 26). In other *Campylobacter* spp including *C. coli*, *C. upsaliensis*, and *C. lari*, there is a co-occurrence of *heuR* and *$_{cj}$ridA* in the genome, suggesting *$_{cj}$ridA* may be regulated in response to differing environmental conditions in these organisms.

The $_{Cj}$RidA has been consistently misannotated as an endoribonuclease in *C. jejuni* studies, obscuring its likely connection to PLP-dependent enzymes and metabolism. In *S. enterica*, RidA catalyzes the hydrolysis of the 2AA intermediate formed by several PLP-dependent enzymes (3, 14, 15). In the absence of RidA, free 2AA accumulates and can covalently inactivate certain PLP-dependent enzymes such as serine hydroxymethyltransferase (SHMT) (GlyA; EC 2.1.2.1), alanine racemases (Alr/DadX; EC 5.1.1.1), and transaminase B (IlvE; EC 2.6.1.42), leading to defects in one-carbon unit metabolism, cell-wall synthesis, and isoleucine biosynthesis, respectively (10, 13, 27). The activity of the enzymes targeted by 2AA can be decreased by 30-50% in strains lacking RidA (8, 9, 16, 17, 27). To date, the diverse phenotypes of organisms lacking RidA suggest that there are additional and unknown targets of 2AA, possibly extending beyond PLP-dependent enzymes.

In at least *S. enterica*, *E. coli*, *P. aeruginosa*, *S. cerevisiae,* and *Arabidopsis thaliana,* a biosynthetic threonine/serine dehydratase (IlvA), acting on serine is the main source of 2AA in *ridA* mutants (3, 9, 16, 28, 29). In each of these organisms, IlvA has a regulatory domain and is allosterically inhibited by isoleucine (10, 30). As a consequence, the presence of isoleucine eliminated generation of 2AA and suppressed the phenotypes of a *ridA* mutant. The specific targets of 2AA that result in detectable defects vary in different organisms. In *S. enterica*, 2AA accumulation causes a growth defect reversed by exogenous glycine; in *E. coli* 2AA accumulation-induced growth inhibition was reversed by exogenous aspartate, or purines; in *P. aeruginosa*, 2AA accumulation is detrimental to growth and partially reversed by exogenous proline and polyamines; and in *S. cerevisiae* mitochondrial accumulation of 2AA leads to loss of mitochondrial DNA and reduced heme biosynthesis (9, 13, 16, 17, 27) (Whitaker and Downs, unpublished). The

diverse effects of 2AA emphasize the complexity of the metabolic network and our limited understanding of the integration between biochemical pathways.

This study was initiated to understand the physiological role of $_{Cj}$RidA (Cj1388) in *C. jejuni* 11168. Although the levels of *$_{cj}$ridA* were noted in multiple global studies, this gene was peripheral in those studies, and in many cases the results were not verified nor was the effect determined to be direct or indirect. The data herein suggest a role for $_{Cj}$RidA in flagellar biosynthesis, structure, glycosylation, and/or function. Further, we confirmed that $_{Cj}$RidA and the Rid2 subfamily member Cj0327, have enamine deaminase activity *in vivo* and *in vitro*. This work extends the list of organisms known to encode functional enamine deaminases of the Rid family.

3.3 Materials and Methods

Bacterial strains, plasmids and media. The strains, plasmids and primers used in this study are listed in Table 1, 2, and 3 with their sources. *C. jejuni* human isolate NCTC 11168 (31) was used as the parental strain. Derivatives of *S. enterica* serovar Typhimurium LT2 (*S. enterica*) were used for *in vivo* complementation studies.

Derivatives of *C. jejuni* 11168 were grown on Mueller Hinton (MH, 21 g/liter), Brain heart infusion (BHI, 37 g/L), Brucella agar (28.1 g/L) or NZCYM (22 g/L) at 37 ˚C under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) (32). *S. enterica* and *E.* coli strains were grown in Difco Nutrient Broth (8 g/l) with NaCl (5 g/l) at 37°C. Minimal medium was NCE salts with $MgSO_4$ (33), trace minerals (34), and 11 mM glucose. Additions, isoleucine (1 mM), and serine (5 mM) were added as indicated. Antibiotic concentrations were as follows; 150 µg/mL ampicillin or 50 µg/mL kanamycin were used for *S. enterica* and 15 µg/mL chloramphenicol or 30 µg/mL kanamycin were used for *C. jejuni*. When needed to induce expression of genes in relevant

plasmids, L-arabinose was added (0.2%). Chemicals were purchased from MilliporeSigma (Sigma-Aldrich, St. Louis, MO).

Growth quantification. Growth of *S. enterica* in liquid culture was assessed using a BioTek Elx808 microtiter plate reader following optical density at 650 nm at 37˚C with slow shaking speed. Overnight cultures of *S. enterica* in biological triplicate were grown in rich medium at 37° C, pelleted and resuspended in an equal volume of sterile NaCl (8.5 g/L). The resulting cell suspension was used to inoculate growth medium (2% inoculum) and growth was monitored for 24 hours. The resulting data were plotted using GraphPad Prism 7.0, generating curves in log10-format that display the mean of three replicates and standard deviation of the mean. Specific growth rates (μ) were calculated according to the following equation: $\ln(X/X_0)/T$, where X is OD_{650}, X_0 is the starting OD_{650} of the exponential growth period monitored, and T is time in hours.

Molecular biology. A plasmid (pCASO29) with a deletion/ kanamycin insertion construct in *cj1388* was used to construct a $_{cj}$*ridA*::kan mutant (DMC3, DMC4, and DMC5) (18). A *pseC*::kan mutant was obtained from the Szymanski laboratory collection. Additional mutants were constructed using standard methods (35, 36). Briefly, to generate an insertion/deletion in a gene of interest, homology both up- and down-stream to the gene of interest was joined to a drug resistance cassette by overlap extension PCR. PCR products were purified using Qiagen gel extraction kit (ID 28506). The natural competence of *C. jejuni* was exploited to transform the PCR product into cells grown on nutrient rich medium, BHI with 2% yeast extract. After 24 hours growth, cells were streaked on selective medium and colonies formed after 3-5 days. Colonies were streaked for isolation and culture stocks were frozen in glycerol. Insertion deletions of relevant genes was confirmed by PCR.

Derivatives of plasmid pBAD24 and pET28b were created using a BspQI restriction cloning method as previously described by Galloway *et al*. with a modified vector that contained the BspQI site (pCV1) (37, 38). *S. enterica* or *E. coli* competent cells were prepared and transformed using standard method. Transformants were recovered in nutrient broth, plated to selective medium at 37°C before isolating and confirming the plasmid structure.

For *pseC*::km mutant construction, *pseC* was amplified from 11168 using the *pseC*-F and *pseC*-R primers (Table 4). This insert was purified, digested with BamHI and XhoI, inserted into PCRscript (Stratagene) digested with the same enzymes, and transformed into *E. coli* DH5α. The extracted plasmids were digested with XhoI and BamHI to confirm insert presence and one plasmid subsequently digested with NcoI, purified, blunted and treated with alkaline phosphatase to prevent re-ligation. The *pseC* gene was interrupted by ligating a kanamycin resistance cassette (km). The mutant was confirmed using the *pseC*-F/mid KmR primer pair (Table 4) and sequenced.

Protein Production and purification. Proteins $_{Cj}$RidA and Cj0327 were purified from *E. coli* strain BL21AI harboring pET vector constructs. The polyhistidine-tagged proteins were purified by nickel-affinity chromatography as previously described (8). Overnight cultures in LB (10 mL) were used to inoculate flasks containing super broth (1.5 L) supplemented with kanamycin (50 µg/mL). Cultures were grown at 37˚C with shaking until an OD_{650} between 0.7 and 1.0 was reached. Arabinose (0.2%) was added to induce T7 RNA polymerase for 18 hours. Cells were harvested by centrifugation at 7,000 x g and the cell 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 One Shot Cell Disruptor at 18,000 psi (Constant Systems). The lysate was clarified by centrifugation (40,000 x g for 45 minutes) and

passed through a 0.45 μm syringe filter (Argos Technologies) prior to being loaded onto 5 mL HisTrap™ HP column and purified using the manufacturer's protocol (GE Healthcare). 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). The fractions were assessed for purity, pooled, and concentrated using a 7,000 molecular weight cut-off protein concentrator (Millipore). The protein preparations were dialyzed into storage buffer (50 mM potassium phosphate pH 7.5, 10% glycerol) using a PD-10 desalting column (GE Healthcare). Proteins were subjected to SDS/PAGE and purity was assessed using a Foto/Analyst FX (Fotodyne) imager and TotalLab Quant v11 densitometry software. Protein concentration was quantified using BCA Protein Assay (Thermo Scientific), and the samples were frozen in liquid nitrogen and stored at -80˚C.

L-amino acid oxidase assays. The LOX-based assay for Rid activity was adapted from a previously described assay and has been used to assess activity of Rid proteins from several organisms (5, 39-41). The 2-iminobutyrate intermediate from 2-aminobutyrate was derivatized with semicarbazide resulting in semicarbazone detected by absorbance at 248 nm. The assay mixture (100 μL total volume) contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), bovine liver catalase (1 μg), L-amino acid oxidase (0.5 μg) and 0.1, 1.0 or 10 μM Rid protein. Reactions were started in a 96-well quartz plate with the addition of 2-aminobutyrate to the final concentration of 0.5 mM. Following the addition of substrate, the path length for each well was measured and used along with the molar extinction coefficient for semicarbazone (ε = 10,300 M^{-1} cm^{-1}) to calculate the rate of semicarbazone formation. Standard deviation of the mean was determined from three technical triplicates by GraphPad Prism 7.0c.

Motility. Assays for swimming motility were done by modifying previously described methods (42-45). Briefly, bacteria were harvested from overnight growth on BHI or MH agar plates into PBS and the OD_{600} was adjusted to 1.0. Ten µL of the bacteria suspension was inoculated on individual plates by gently piercing the soft agar before expelling the cell suspension into 0.4% agar Brucella, BHI, or MH. Agar plates were incubated at 37 ˚C for 24-72 hours. The diameter of each swimming halo was measured and recorded in millimeter (mm). A non-motile *pseC* mutant served as a negative control; the spread of the *pseC* inoculum was subtracted from the motility zone diameter of the experimental strains and the number divided by 2 to get the motility distances as reported in mm. The data shown represent the mean of three technical replicates. For each mutation of interest, three independently isolated mutants were tested to ensure phase variability did not contribute to motility defects. Error bars represent the standard error of the mean. Statistical significance ($P < 0.02$) was determined by unpaired Student's test (t test) using GraphPad Prism 7.0c.

Phage NCTC 12673 plaque assay. Plaque formation by NCTC 12673 phage was tested by spotting dilutions of a lysate onto a freshly inoculated bacterial suspension using a standard agar overlay method (46). Briefly, 100 µL of a bacterial suspension (OD_{600} of ~0.35) was mixed with 5 mL sterile 0.6% molten NZCYM agar (Sigma-Aldrich, St. Louis, MO) and poured onto the surface of a NZCYM plate. After 15 min, 10 µL aliquots of serial dilutions of a phage lysate were spotted onto the agar surface and allowed to completely dry before incubation at 37 ˚C under microaerobic conditions. After 24 hours, plaques were counted, and the apparent number of PFU/mL was determined.

Autoagglutination Assays. Published protocols for autoagglutination were adapted for use (18, 47, 48). Simply, cells were harvested from overnight growth on MH agar plates and resuspended in MH broth. The OD_{600} was measured and adjusted to 1.0 in 5 mL of MH broth with 0.002% Tween-20 in a glass test tube. The top 1 mL was removed and OD_{600} measured (OD_{600i}). The remaining 4 mL sat without shaking at room temperature in air. At 24, and 48 hours, a 1 mL aliquot of the liquid was removed and the absorbance was measured to obtain the recorded OD_{600} (OD_{600r}). The % of autoagglutination (%AAG) reported was calculated as {(OD_{600i} - OD_{600r}) / OD_{600i}} x 100.

3.4 Results

Cj1388* and *Cj0327* deaminate 2-aminoacrylate *in vivo. A *S. enterica ridA* mutant fails to grow on minimal medium with serine due to the accumulation of 2AA that is generated by the biosynthetic serine/threonine dehydratase encoded by *ilvA* (EC 4.3.1.19) (4, 10). A *S. enterica ridA* mutant was transformed with pBAD24 constructs harboring a gene encoding Rid proteins from *C. jejuni* ($_{cj}$*1388*/$_{cj}$*ridA* or *cj0327*), the *S. enterica ridA* ($_{se}$*ridA*) under the control of an arabinose promoter, or an empty vector control. Growth was monitored in minimal glucose medium with 5 mM serine and the data are shown in Figure 1. Plasmids carrying either $_{cj}$*ridA* (pDM1577) or $_{se}$*ridA* (pDM1439) restored full growth to the *S. enterica ridA* mutant without inducing expression of the plasmid encoded genes. In contrast, *cj0327* (pDM1588) partially restored growth, and only when its expression was induced with arabinose.

The inability of Cj0327, a Rid2 subfamily member, to fully complement the *S. enterica ridA* mutant was consistent with the results obtained for proteins annotated as Rid1-3 from *P. aeruginosa*, *Yersinia pestis*, *E. coli*, *Acinetobacter baylyi*, and *Pseuodomonas syringae* (16, 40) (Irons *et al.*, unpublished). The partial complementation by Cj0327 and other proteins from the

Rid1, 2, and 3 subfamilies suggests that these proteins may deaminate primarily non-2AA enamines *in vivo*. The specific physiological role of Cj0327 was not pursued further here.

Cj1388* and *Cj0327 have deaminase activity in vitro. L-amino acid oxidase (LOX or LAAO)-based assays were used to assess the ability of purified Cj1388 and Cj0327 to deaminate imines *in vitro* (5, 39-41, 49). 2-aminobutyrate was provided as substrate, resulting in the LOX-dependent formation of 2-iminobutyrate. This imine reacts with semicarbazide to produce a semicarbazone which is monitored at 248 nm. Rid proteins can compete for the imine, converting it to the ketoacid, 2-ketobutyrate, similar to a reaction RidA catalyzes *in vivo*. Thus in this assay, the rate of semicarbazone formation is inversely proportional to Rid activity. The rate of semicarbazone formation (μM, min^{-1}) with the addition of Cj1388 ($_{Cj}$RidA) or Cj0327 is shown in Figure 2. Consistent with the *in vivo* complementation data in an *S. enterica ridA* mutant, $_{Cj}$RidA has greater deaminase activity than Cj0327. When the Rid proteins are provided at higher concentrations (i.e., 10 μM), semicarbazone formation is reduced to the same extent by each protein, consistent with a saturating concentration of enzyme.

***Campylobacter jejuni* mutants lacking $_{cj}$*ridA* have a motility defect.** Data from several bacterial species suggested RidA is involved in flagellar biosynthesis and/or motility (16, 18, 28). A variant of *C. jejuni* 11168 lacking $_{cj}$*ridA* was generated and assessed for swimming motility on Mueller Hinton (MH) medium with 0.4% agar and 0.01% Triphenyltetrazolium Chloride (TTC). The data (Figure 3) showed that the $_{cj}$*ridA* mutant had significantly decreased motility when compared to wild type over the course of 72 hours. A non-motile, aflagellate *pseC* mutant was used as a control to determine the spread of the inoculum that was due to diffusion. The motility data in Figure 3 is representative of experiments that were performed more than ten times on ten different days and included three independently constructed $_{cj}$*ridA* mutants. Although there was

day-to-day variation in the absolute motility measured, the difference between the mutant and control strains remained consistent at ~2-fold. Motility was not affected by a lesion in the gene encoding the Rid2 subfamily member (*cj0327*) in either a wild-type background or the $_{cj}$*ridA* mutant, (data not shown). This result supported the conclusion that the $_{Cj}$RidA and Cj0327 proteins are not physiologically redundant in *C. jejuni*, consistent with what has been found in other organisms. Addition of isoleucine (1 mM) to the motility agar did not affect the motility defect of the $_{cj}$*ridA* mutant (data not shown), and motility was restored to wild-type levels when $_{cj}$*ridA* was inserted into pseudogene *cj0046* and expressed with its native promoter (Figure 4).

A previous study reported $_{cj}$*1388* mutants had increased motility compared to wild-type in Brucella motility agar, a rich undefined medium (18). Motility was assessed in Brucella, brain heart infusion (BHI), and MH motility agar for two mutants and wild type and the data are in Figure 4. Both Brucella and BHI media support increased mobility (and growth) of both mutants and wild type. Similar to what was previously reported, $_{cj}$*ridA* mutant motility was 1.1-fold and 1.4-fold higher than wild-type in BHI and Brucella motility media, respectively. Significantly, the $_{cj}$*ridA* mutant displayed a motility defect only on MH medium. The restoration of motility on the two complex medium is consistent with regulation of metabolic flux in $_{cj}$*ridA* mutants limiting the production of, and/or damage by, the reactive enamine substrate of the $_{Cj}$RidA protein. For instance, in *S. enterica* and *P. aeruginosa ridA* mutant motility defects only arise when minimal defined media is used and the defects are eliminated by the addition of isoleucine which prevents 2AA formation. Given the complexity of metabolic systems and regulation, minimal defined media will be used in future studies to determine the impact of $_{cj}$*ridA* mutations motility.

$_{Cj}$RidA is required for full infection and/or lysis by phage NCTC 12673. The *C. jejuni* lytic phage, NCTC 12673 has decreased plaquing efficiency on aflagellate mutants (46) and fails to form plaques on *pseC* mutants (50). Plaque formation by NCTC 12673 was assessed with serially diluted aliquots of a phage lysate spotted on 0.6% agar overlays seeded with the indicated mutant or wild type. The efficiency of plating was tested on wild-type *C. jejuni*, a *$_{cj}$ridA* mutant (Figure 5) and a *pseC* mutant. As expected, no plaques were visible on the *pseC* mutant, which lacks the ability to synthesize pseudaminic acid, the major glycan modification of FlaA and FlaB subunits of the flagellum, and is therefore aflagellete (50, 51). When plated on wild-type *C. jejuni*, the phage titer was 1 x 10^7 PFU/ml. When the same lysate was plated on a *$_{cj}$ridA* mutant, the titer was 2 x 10^6. This approximately 5-fold decrease in plating efficiency compared to the parental strain was consistent with the hypothesis that the *$_{cj}$ridA* mutant had a defect in flagellar biosynthesis and/or function.

***$_{cj}$ridA* mutants have a defect in autoagglutination.** The decreased motility and sensitivity to phage NCTC 12673 suggested a flagellar defect in the *$_{cj}$ridA* mutants. In both cases, the *$_{cj}$ridA* mutant phenotype fell between that of the wild type and the *pseC* mutant, which completely lacks flagella. Consistently, a hallmark of *ridA* mutants is the decreased, but not eliminated activity of the enzymes targeted by 2AA causing phenotypes that are less severe than complete lesions of the relevant enzymes. Changes in autoagglutination (AAG) can also indicate a change in flagella, specifically in flagellar glycan decoration, which correlates with a reduction in virulence (48, 52, 53). Reuter *et al*., reported that a *cj1388* (*$_{cj}$ridA*) mutant had a slower rate of AAG compared to wild-type *C. jejuni* 11168, in medium supplemented with Tween-20 (0.002%) (18). AAG was determined in our hands for wild type and *$_{cj}$ridA* mutant after suspension in several different media. Cells were harvested from MH agar plates and suspended in MH or PBS as appropriate. The cell

suspension was adjusted to an OD_{600} of 1.0 in 5 mL of: i) MH, ii) MH with 0.002% Tween-20, or iii) PBS. Consistent with previous observations, the *$_{Cj}$ridA* mutant had a significant and reproducible decrease in AAG compared to wild type in MH supplemented with 0.002% Tween-20, reflected by more cells remaining in suspension (Figure 6). Each mutant was tested in triplicate. To ensure that any observed difference in phenotype was due to specific mutation, three separate clones for each mutant were tested separately and then the data were combined. The defect of a *$_{Cj}$ridA* mutant appeared to reflect a slower rate of AAG, since the defect was significant after a 24-hour incubation, but by 48 hours the mutants were not significantly different than wild type. In our hands, other media used in reported AAG protocols (PBS or MH alone) failed to result in visible differences between the mutant and wild type. As expected, a *pseC* mutant showed almost complete cessation of AAG, thus another phenotype of the *$_{Cj}$ridA* mutant fell between that of a wild type and the *pseC* mutant (Figure 6). The decreased rate of autoagglutination in a *$_{Cj}$ridA* mutant supported the emerging model that $_{Cj}$RidA directly or indirectly affected flagellar regulation, biogenesis, glycosylation, or structure.

Transmission electron microscopy shows $_{Cj}$RidA impacts flagella. Transmission electron microscopy (TEM) was performed on cells harvested from MH agar plates and suspended in PBS (48). Efforts to fix cells with glutaraldehyde and formaldehyde or paraformaldehyde and stain with uranyl acetate or phosphotungstic acid failed to yield clear images and thus the cells were imaged with no fixative or stain (Figure 7B). The number of flagella were quantified using two independently constructed *$_{Cj}$ridA* mutants and a wild-type strain of *C. jejuni* (Figure 7A). One hundred cells with two unobstructed poles from each mutant and the wild type were used for quantification. Of the one hundred wild-type cells observed, ~ 60% had bipolar flagella, ~20% had a single flagellum, and ~20% had no visible flagellum. In contrast, of the 200 *$_{Cj}$ridA* mutant cells

observed, 20% had bipolar flagella, <40% had a single flagellum, <40% had no flagella. Beyond the number, structural anomalies of the flagella were noted in the mutant cells that were not seen in the wild-type sample (Figure 7B). First, there were "nub" structures on one or both poles of the bacterium (~10% of mutant cells). Secondly, there were instances where flagella in the mutant were unusually long and apparently thinner than the wild type. Together these observations showed that the lack of $_{Cj}$RidA significantly impacted flagellar synthesis and or assembly. TEM images do not provide clarity on the specific flagellar defect caused by a *$_{cj}$ridA* mutation. Regardless, the images, in combination with the phenotypic analysis above allowed the conclusion that $_{Cj}$RidA is important for the full formation of a functional flagella.

Cj0828 is the biosynthetic serine/threonine dehydratase in *C. jejuni.* In five organisms previously characterized, the phenotypic effects of eliminating the RidA homolog were due to the accumulation of 2AA, generated by a PLP-dependent serine threonine dehydratase enzyme (EC 4.3.1.19). As a consequence, a hallmark of the paradigm thus far has been the suppression of all defects by exogenous isoleucine, which allosterically inhibits the dehydratase enzyme(s). Within this context, it was striking that phenotypes associated with a *$_{cj}$ridA* mutation in *C. jejuni* were apparent in nutrient (MH) medium that contained abundant isoleucine. *C. jejuni* encodes a single gene annotated as a PLP-dependent serine/threonine dehydratase, (Cj0828, EC 4.3.1.19). Cj0828 shares 32% identity to *S. enterica* IlvA (Figure 8) but it lacks the C-terminal domain that contains the allosteric site for inhibition by isoleucine (54, 55). These data suggested that if *cj0828* encoded the legitimate biosynthetic threonine dehydratase, the presence of isoleucine would not prevent generation of 2AA by this enzyme. An insertion deletion was introduced into *cj0828* and growth was tested on a defined minimal medium (MCLMAN). In minimal medium, the *cj0828* mutant required isoleucine for full growth, indicating this gene product was the biosynthetic

serine/threonine dehydratase *in vivo* (data not shown). To reflect this result, the gene was renamed *$_{cj}$ilvA*. The identification of *$_{cj}$ilvA* suggested three possible scenarios to explain the RidA paradigm in *C. jejuni*: 1) *$_{cj}$ilvA* is constitutively expressed and thus generates 2AA even on nutrient medium, 2) there are other enzyme(s) in the cell that generate 2AA, or 3) 2AA is not responsible for the phenotypes of the *$_{cj}$ridA* mutant. The latter would suggest there was another reactive metabolite produced in the cell that is quenched by $_{Cj}$RidA.

***C. jejuni* expands features of the RidA paradigm.** The contribution of $_{Cj}$IlvA to the phenotypes of a *$_{cj}$ridA* mutant was tested by constructing a double mutant. A *$_{cj}$ilvA* loss of function mutation was introduced into a $_{cj}$*ridA* mutant background and the resulting double mutant was assessed for motility. Motility assays were performed with the *$_{cj}$ridA $_{cj}$ilvA* double mutant on MH with 0.4% agar (Figure 9). The motility of the *$_{cj}$ilvA* mutant was indistinguishable from the parental wild type. Similarly, the motility of the *$_{cj}$ridA $_{cj}$ilvA* double mutant was no different than the single *$_{cj}$ridA* mutant. Importantly, both mutants carrying a *$_{cj}$ridA* mutation had a >2-fold decrease in motility compared to their respective parental strain. These data showed that $_{Cj}$IlvA was not the source of sufficient 2AA to result in the phenotypes detected for the *$_{cj}$ridA* mutant on MH motility agar. Thus, the source of the reactive enamine presumed to be responsible for the flagellar defects of the *$_{cj}$ridA* mutants remains to be determined.

3.5 Discussion

The data herein demonstrate that the gene designated *cj1388* in *Campylobacter jejuni* 11168 is a RidA with 2AA deaminase activity *in vivo*. *C. jejuni* is the first organism to date where the major phenotypic consequences of lacking RidA are not caused by the activity of a serine threonine dehydratase. Thus *C. jejuni* provides an opportunity to identify additional generators of reactive enamine(s) like 2AA, that can impact the physiology of different organisms in the absence

of RidA. One of the two additional 2AA generators found in *S. enterica,* cysteine desulfhydrase (CdsH; EC 2.5.1.47), appears to be present in *C. jejuni* and additional work will determine if this enzyme has a role in generating the phenotypes of a $_{cj}$*ridA* mutant.

Results presented herein, which used three independent $_{cj}$*ridA* mutants, suggest *C. jejuni* 11168 lacking *ridA* has a defect in flagellar biosynthesis, regulation, or structure. *C. jejuni* mutants lacking $_{cj}$*ridA* have defects in motility, AAG, and phage infectivity, all of which require or are enhanced by flagella (42, 51, 56). Motility is essential for *C. jejuni* to move through the viscous mucosal environment to colonize a human host, and protein glycosylation is essential for flagellar biosynthesis and function. Flagellum (FlaA and FlaB) subunits are modified by O-linked pseudaminic and legionaminic acids and their derivatives at up to 19 Ser/Thr sites before export and assembly of the flagellar apparatus (57-61). Importantly, thus far the only defined targets of accumulated 2AA are PLP-dependent enzymes. Given the importance of glycosylation of the flagellar subunits, it is possible that the UDP-4-amino-4,6-dideoxy-N-acetyl-ß--L-altrosamine transaminase (Cj1294/PseC; EC 2.6.1.92), a fold-type II PLP-dependent enzyme, could be a critical target of 2AA and thus be damaged in a $_{cj}$*ridA* mutant.

Our favored model suggests that 2AA accumulates in a $_{cj}$*ridA* mutant and damages PLP-dependent enzyme, PseC, leading to a decrease in pseudaminic acid modification on FlaA. Consistent with this model, changes in FlaA glycosylation affect AAG, motility, and virulence (47, 48, 57-59, 61). Based on other examples, damage by 2AA is expected to reduce the activity of PseC 30-50% (8, 9, 16, 17, 27). In this case, the phenotypes resulting from PseC damage could vary among the cell population and be similar to the range of phenotypes previously shown from *flaA* point mutations (61-63).

3.6 References

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Table 3.1: Strains used in this study.

Organism	Mutant ID	Genotype	Plasmid	Source
S. enterica	DM14846	*ridA1*::Tn10(Tc)	pDM1439	Downs lab
	DM14847	*ridA1*::Tn10(Tc)	pCV1 (Empty vector)	VanDrisse, 2016
	DM16385	*ridA1*::Tn10(Tc)	pDM1577 (*cj1388*)	This study
	DM16513	*ridA1*::Tn10(Tc)	pDM1588 (*cj0327*)	This study
E. coli	DM16869	DH5a	pCASO29 (*cj1388*::kan)	Reuter, 2015
	DM16508	BL21AI	pDM1589 (pET28b_*cj0327*)	This study
	DM16383	BL21AI	pDM1578 (pET28b_*cj1388*)	This study
C. jejuni 11168	DMC1	Wild type	-	Parkhill, 2000
	DMC2	*pseC*::kan	-	Szymanski Lab
	DMC3	*cjridA*::kan A	-	This study
	DMC4	*cjridA*::kan B	-	This study
	DMC5	*cjridA*::kan C	-	This study
	DMC6	*cj0327*::cat A	-	This study
	DMC7	*cj0327*::cat B	-	This study
	DMC8	*cj0327*::cat C	-	This study
	DMC9	*cjridA*::kan *cj0828*::cat A	-	This study
	DMC10	*cjridA*::kan *cj0828*::cat B	-	This study
	DMC11	*cjridA*::kan *cj0828*::cat C	-	This study
	DMC12	*cj0046*::*cjridA*-cat A	-	This study
	DMC13	*cj0046*::*cjridA*-cat B	-	This study
	DMC14	*cj0046*::*cjridA*-cat C	-	This study

Table 3.2: Plasmids used in this study.

Plasmid	Description	Source
pCV1	BspQI modified pBAD24 vector	Downs Lab
pDM1439	*S. enterica ridA* pBAD24	Downs Lab
pDM1577	*C. jejuni cj1388* pBAD24	This study
pDM1588	*C. jejuni cj0327* pBAD24	This study
pDM1589	BspQI modified pET28b_*cj0327*	This study
pDM1578	BspQI modified pET28b_*cj1388*	This study
pCASO29	Gene disruption plasmid *cj1388*::kan	Reuter, 2015

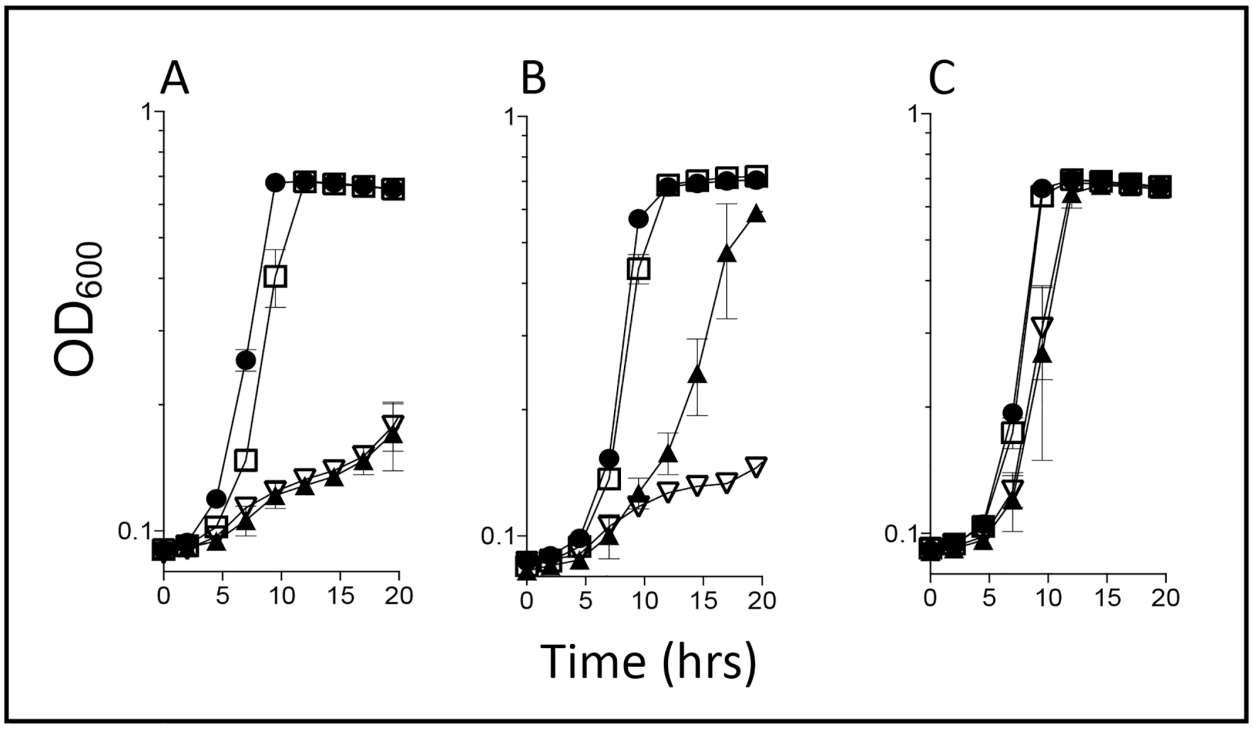
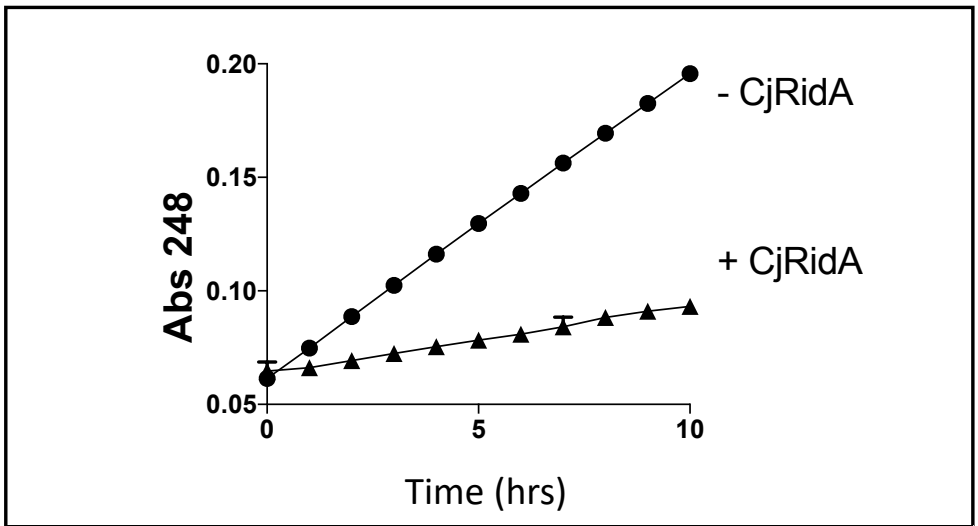


Figure 3.1: *$_{cj}$ridA* complements a S. *enterica ridA* mutant. A *S. enterica ridA* mutant with one of four plasmids was grown in a 96-well plate at 37 ˚C shaking in minimal glucose (11 mM) medium with: (A) serine (5 mM), (B) serine and arabinose (0.2%), or (C) serine, arabinose and isoleucine (1 mM). The *S. enterica* strain carried plasmids expressing *S. enterica ridA* (filled circles), *C. jejuni ridA* (*$_{cj}$ridA*, open squares), *C. jejuni rid*2 (*cj0327*, closed triangles), or empty vector (open triangles).



	Rate of Semicarbazone Formation						
	No RidA	CjRidA 0.1uM	CjRidA 1.0uM	CjRidA 10uM	Cj0327 0.1uM	Cj0327 1.0uM	Cj0327 10uM
(uM, min-1)	1.17 ± 0.10	0.826 ± 0.06	0.266 ± 0.02	0.153 ± 0.06	1.09 ± 0.09	0.686 ± 0.01	0.23 ± 0.03

Figure 3.2: $_{Cj}$RidA and Cj0327 are imine deaminases *in vitro*. Each reaction mixture contained contained potassium pyrophosphate (50 mM, pH 8.7), neutralized semicarbazide (10 mM), bovine liver catalase (1 μg), and L-amino acid oxidase (0.5 μg) with or without the addition of CjRidA or Cj0327. 2-aminobutyrate (0.5 mM) was added to start the reaction and absorbance at 248 nm was monitored for 10 minutes. The graph shows the absorbance over time for reactions without CjRidA (circles) or with CjRidA at a final concentration of 1.0 μM (triangles). Error bars represent standard deviation of the mean determined from three technical triplicates by GraphPad Prism 7.0c. The the molar extinction coefficient for semicarbazone (ε = 10,300 M^{-1} cm^{-1}) was used to calculate the rate of semicarbazone formation (μM,min-1) in reactions without Rid proteins and with $_{Cj}$RidA and Cj0327 in concentrations of 0.1, 1.0, and 10 μM. Standard deviation of the mean was determined from three technical triplicates by GraphPad Prism 7.0c

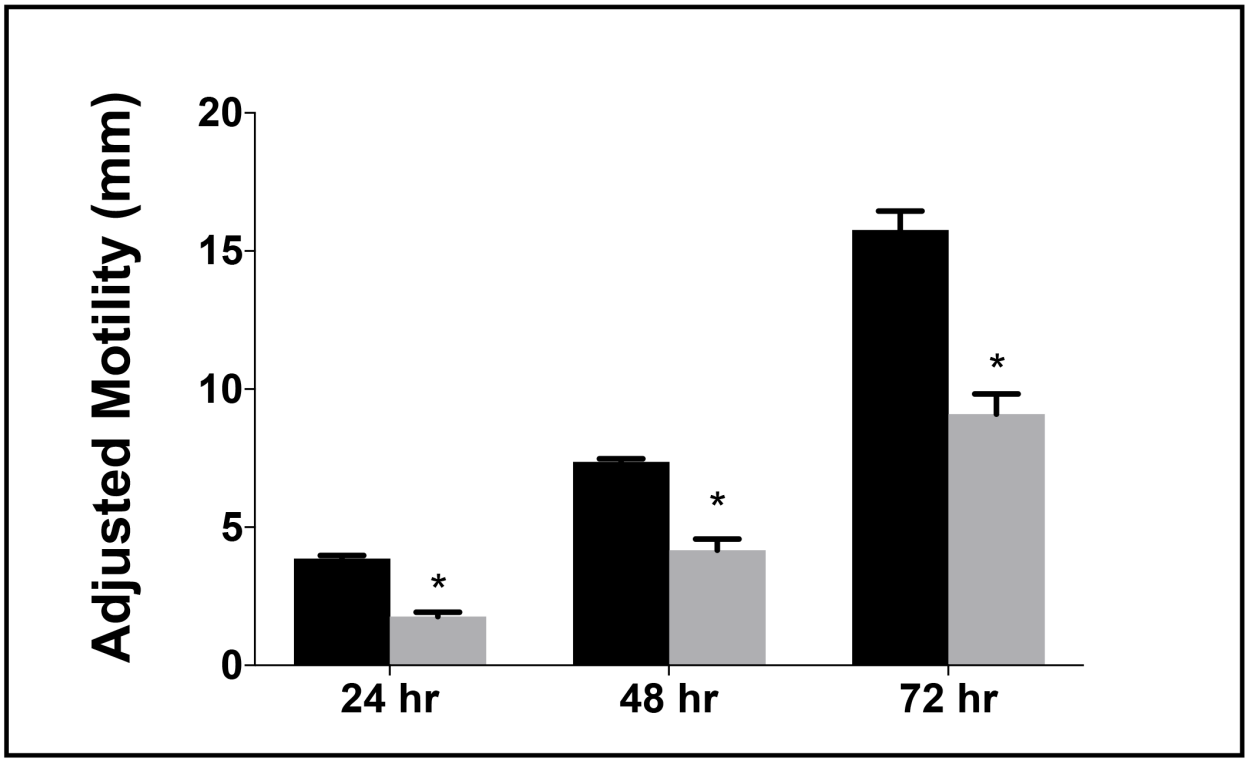


Figure 3.3: *C. jejuni ridA* mutants have a significant defect in motility. Swimming motility was determined for *C. jejuni* wild type (black) and a $_{cj}$*ridA* mutant (gray). Ten µL of cell suspension was inoculated in the center of MH agar (0.4%) plate that was incubated up to 72 hours in microaerophilic conditions. Motility was defined as swimming-dependent spread by subtracting the diameter of inoculum diffusion from the motility zone and dividing it by two. Error bars represent the standard errors of the mean of three technical triplicates, for wild type, and two biological replicates in technical triplicate for the $_{cj}$*ridA* mutant. Significance was determined between wild type and $_{cj}$*ridA* mutant for each time period and an asterisk denotes statistically significant (P<0.005) variation between mutants, as determined by an unpaired Student *t* test performed with GraphPad Prism software, v7.0C.

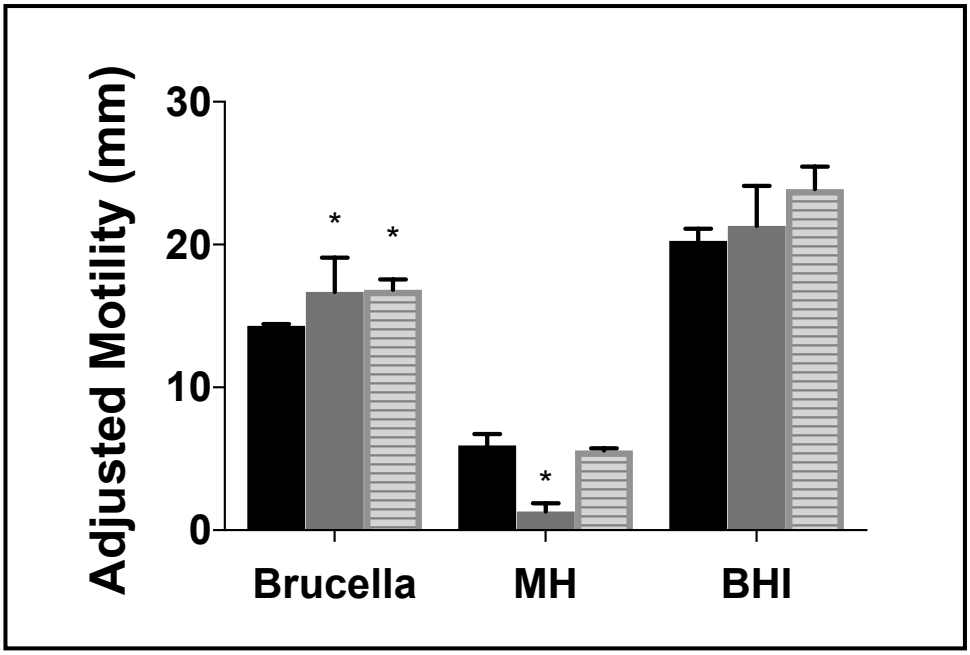


Figure 3.4: Motility of *C. jejuni ridA* mutants differs with media composition. Growth (not shown) and motility were improved by complex undefined-media. (A) Motility of the *C. jejuni* wild type (black), $_{cj}$*ridA* mutant (gray), or the chromosomally complemented $_{cj}$*ridA* mutant ($_{cj}$*0046*:: $_{cj}$*ridA*-kan) (striped) was improved in Brucella or BHI as compared to MH motility agar (0.4%). Ten µL of cell suspension was inoculated in the center of each agar (0.4%) plate that was incubated up for 36 hours in microaerophilic conditions. Motility was defined as swimming-dependent spread by subtracting the diameter of inoculum diffusion from the motility zone and dividing it by two. Error bars represent the standard errors of the mean of three technical triplicates of wild-type and three biological replicates of $_{cj}$*ridA* and $_{cj}$*0046*:: $_{cj}$*ridA*-kan. Significance was determined between wild type and $_{cj}$*ridA* mutant for each time period and an asterisk denotes statistically significant (P<0.02) variation between mutants, as determined by an unpaired Student *t* test performed with GraphPad Prism software, v7.0C.

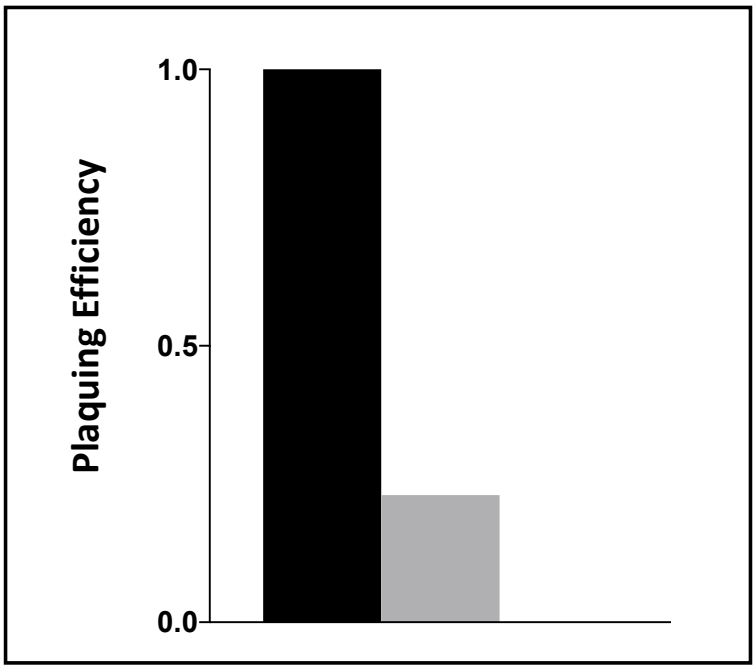


Figure 3.5: *$_{cj}$ridA* mutants support reduced plaque formation of phage NCTC 12673. A phage lysate was titered on *C. jejuni* wild type (black), a *$_{cj}$ridA* mutant (gray) and *pseC* mutant (plaquing efficiency of zero). Serial dilutions of the lysate were spotted on a NZCYM 0.6% agar overlay seeded with the appropriate bacterial mutant. After one day, plaque forming units (PFU/mL lysate) were determined with three technical triplicates of wild type, and two biological replicates in technical duplicate for the *$_{cj}$ridA* mutant. Number of plaques on wildtype was defined as an efficiency of 1.

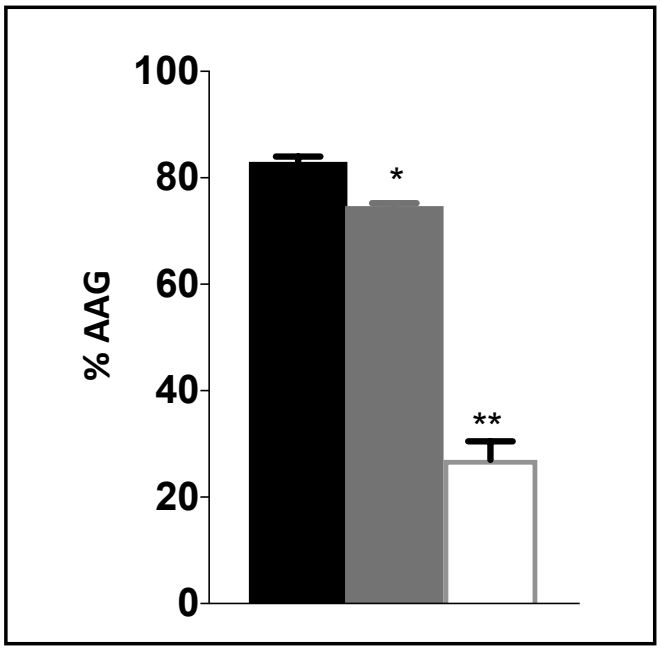


Figure 3.6: *C. jejuni ridA* mutants have an autoagglutination defect during the first 24 hours. Autoagglutination was measured using *C. jejuni* wild type (black), a $_{cj}$*ridA* mutant (light gray) and a *pseC* mutant (white). % AAG represents the percentage of cells that autoagglutinated and settled in the bottom of the tube after 24 hours, determined by the formula $\{(OD_{600i} - OD_{600r}) / OD_{600i}\}$ x 100. Error bars represent the standard errors of the mean of three technical triplicates, for wild type and *pseC* mutant, and two biological replicates in technical duplicate for $_{cj}$*ridA* mutants. Significance was determined between each mutant and wild type. One asterisk denotes statistically significant (P<0.005) and two asterisks denote statistically significant (P<0.0005) variation between mutants, as determined by an unpaired Student *t* test performed with GraphPad Prism software, v7.0C.

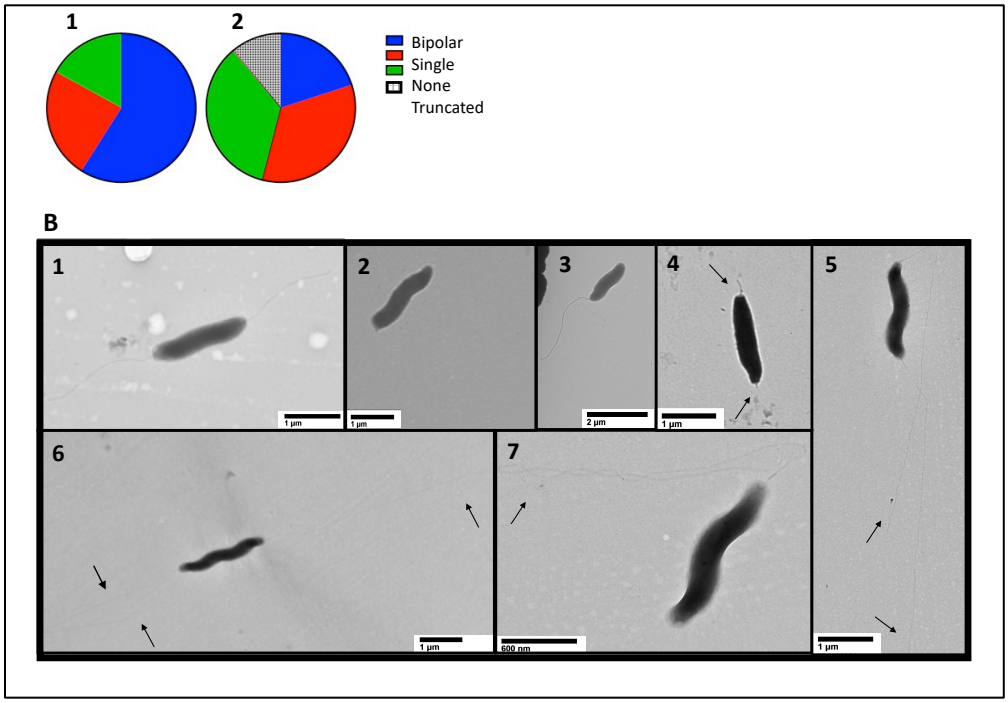


Figure 3.7: TEM detects flagellar differences in $_{cj}$*ridA* mutants. TEM was used to visualize the flagella of wild type and two $_{cj}$*ridA* mutant*s* on multiple days. One hundred cells with clearly visible poles were assessed in each mutant or wild type. Pie charts in (A) represent the distribution of bipolar flagella (blue), a single polar flagellum (red) no flagellum (green) and truncated flagella (hatched). For wild type, N=100, for $_{cj}$*ridA*, N=200 (with 100 from each of two independent mutants) and long and thin flagella (quantified as bipolar or single) were classified by number of flagellar filaments. Lower panels (B1-7) show representative TEM images for cells with: (B1) bipolar flagella, wild type is represented: (B2) no flagellum, $_{cj}$*ridA* mutant is represented; (B3) a single polar flagellum, $_{cj}$*ridA* mutant is represented; (B4) truncated flagella, seen only in $_{cj}$*ridA* mutants; and (B5-7) long, potentially thin flagella, seen only in $_{cj}$*ridA* mutant.

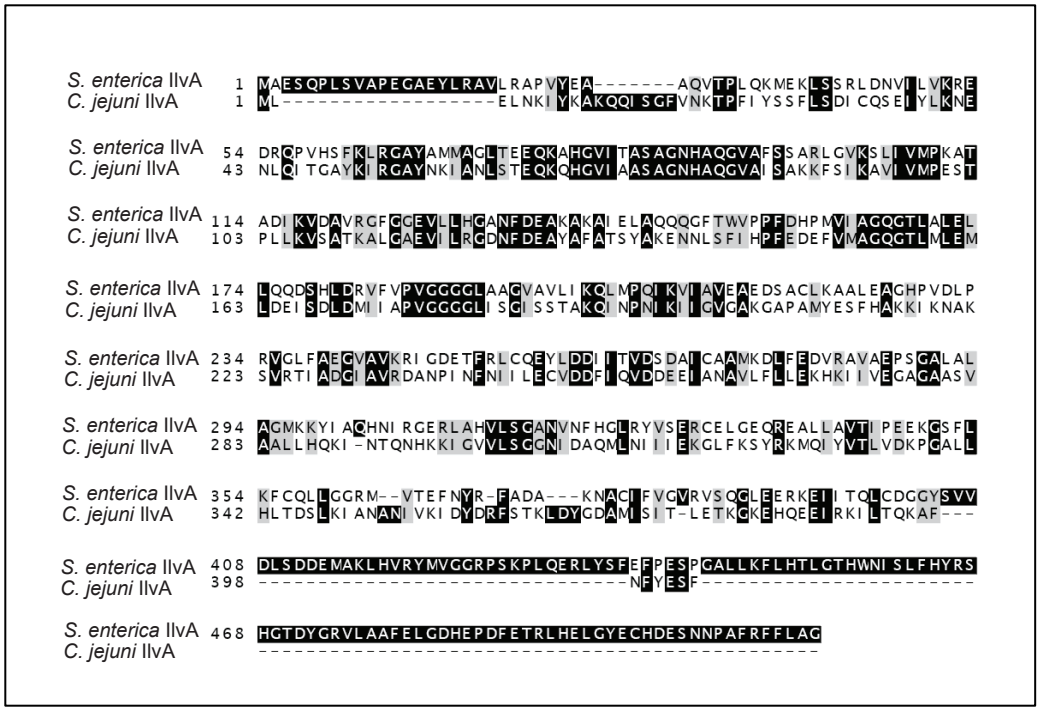


Figure 3.8: *C. jejuni* Cj0828 is an IlvA homolog. *S. enterica* IlvA and *C. jejuni* Cj0828 protein sequences share 32% identity. *Cj0828* ($_{cj}$IlvA) lacks the C-terminal domain that contain the site of allosteric regulation by isoleucine.

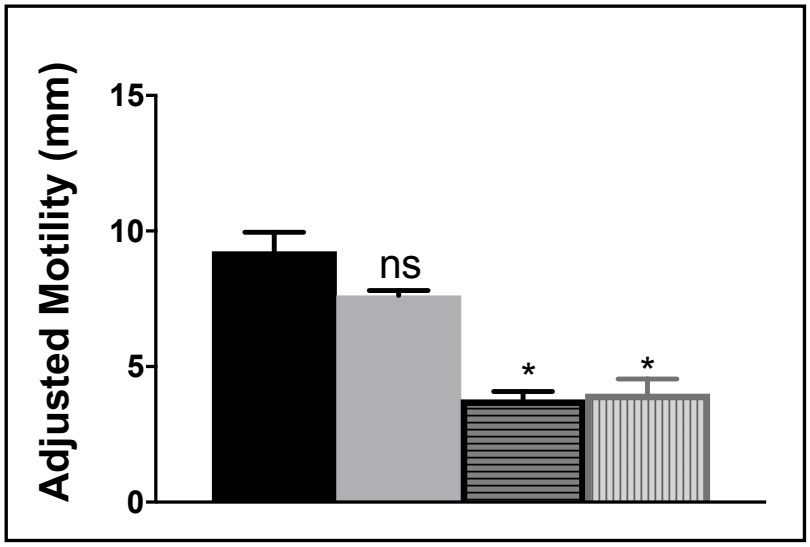


Figure 3.9: A *$_{cj}$ilvA* mutation does not eliminate the phenotype of a *$_{cj}$ridA* mutant. Mutants were grown on BHI overnight. Then cells were scraped from agar surface, resuspended in 1 mL of PBS and the OD_{600} was set to 1.0. Then, 10 µL of cell suspension was used to inoculate the center of a MH 0.4% agar plate. After 48 hours the diameter of motility was measured and the values adjusted to account for the swimming-independent spread of the inoculum, as determined by the non-motile *pseC* mutant. The wild-type parental strain (black) and the *ilvA*::cat mutant (gray) had no defect in motility. The *$_{cj}$ridA* mutant (horizontal stripe) had a >2-fold decrease in motility that was not restored in a *$_{cj}$ridA ilvA* double mutant (vertical stripe). Error bars represent the standard errors of the mean of three technical triplicates, for wild type, and two biological replicates in technical triplicate for *$_{cj}$ridA* and *$_{cj}$ridA cj0828* mutants. Significance was determined between each mutant and wild type asterisk denotes statistically significant (P<0.0001) variation between mutants, as determined by an unpaired Student *t* test performed with GraphPad Prism software, v7.0C.

CHAPTER 4

PA5339, A RIDA HOMOLOG, IS REQUIRED FOR FULL GROWTH IN *PSEUDOMONAS AERUGINOSA*

[1]Irons, JL, [1]Hodge-Hanson KM, [1]Downs DM. 2018. Journal of Bacteriology
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4.1 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. Mutants of *Salmonella enterica*, *Escherichia coli*, and *Saccharomyces cerevisiae* that lack a functional RidA exhibit growth defects, suggesting 2AA metabolic stress is similarly conserved. The PubSeed database shows *Pseudomonas aeruginosa* (PAO1) encodes eight members of the Rid superfamily. Mutants of *P. aeruginosa* PAO1 lacking each of five Rid proteins were screened, and the mutant phenotypes that arose in the absence of PA5339 were dissected. A PA5339::Tn mutant has growth, motility and biofilm defects that can all be linked to the accumulation of 2AA. Further, the PA5339 protein was demonstrably a 2AA deaminase *in vitro* and restored metabolic balance to a *S. enterica ridA* mutant *in vivo*. The data herein showed that the RidA paradigm in *Pseudomonas aeruginosa* had similarities to those described in other organisms, but was distinct in that deleting only one of multiple homologs generated deficiencies. Based on the collective data presented here in, PA5339 was renamed RidA.

4.2 Significance

RidA is a widely conserved protein that prevents endogenous metabolic stress caused by 2-aminoacrylate (2AA) damage to PLP-dependent enzymes in prokaryotes and eukaryotes. The framework for understanding the accumulation of 2AA and its consequences have largely been defined in *Salmonella enterica*. Herein we show that in *P. aeruginosa* (PAO1), 2AA accumulation leads to reduced growth, compromised motility, and defective biofilm formation. This work expands our knowledge how the metabolic architecture of an organism contributes to the

consequences of 2AA inactivation of PLP-dependent enzymes and identifies a key RidA protein in *P. aeruginosa*.

4.3 Introduction

Members of the of the Rid (YjgF/YER057c/UK114) protein superfamily are found in all domains of life and prokaryotic genomes often encode multiple members. The superfamily has been divided into eight subfamilies based on bioinformatic and phylogenetic analysis (1), but it is unclear if these divisions reflect biochemical differences (2). RidA, reactive intermediate deaminase A, the archetypal protein of the family has been primarily studied in *Salmonella enterica* for its role in quenching the reactive metabolite 2-aminoacrylate (2AA), a catalytic intermediate in a number of pyridoxal 5'-phosphate (PLP)-dependent reactions (Figure 1) (3-5). RidA 2AA deaminase activity requires an active site arginine reside (Arg105 in *S. enterica*), and no other residues have been found to be essential for this activity (3). To date, all RidA proteins tested with an active site Arg residue, including human, plant, and archaeal homologs, have demonstrated 2AA deaminase activity *in vivo* and *in vitro* (6, 7). Some members of Rid subfamilies 1-3 also have 2AA deaminase activity *in vivo* and *in vitro*, suggesting at least a partially conserved function of members of the superfamily (2).

Many prokaryotes encode a RidA homolog(s) in addition to one or more representatives from other Rid subgroups (Rid1-7). The presence of multiple Rid proteins in a prokaryotic genome suggests these proteins have distinct roles *in vivo* and could use diverse substrates important to the metabolism of the relevant organism. Efforts in *S. enterica* have laid the groundwork to dissect both the function of different Rid proteins and the role of 2AA on diverse metabolic networks. *S. enterica* encodes one RidA protein and two additional Rid proteins that do not quench 2AA (2). YoaB, a Rid2 subfamily member, hydrolyzes imines produced from FAD-dependent enzymes *in*

vitro, but neither its role *in vivo*, nor its true substrate is known (1). The third Rid protein, STM1549, lacks an active site Arg residue and does not deaminate imines. *E. coli* K12 encodes three Rid proteins, including two RidA homologs that have 2AA deaminase activity: RidA and TdcF (8). Assessing the RidA paradigm in numerous organisms highlighted conserved features and uncovered distinct properties that reflect the consequence of specific metabolic architectures. A *ridA* mutation in *S. enterica* causes a number of growth defects, while growth is unaffected in an *E. coli* double mutant (*ridA*, *tdcF*) unless 2AA accumulation is artificially increased (8). In yeast, inactivating the mitochondrial RidA homolog (Mmf1) results in significant growth and biochemical defects, while loss of the cytoplasmic homolog (Hmf1) fails to have detectable consequences (9-11).

In *S. enterica*, the PLP-dependent enzymes threonine/serine dehydratase (IlvA; EC 4.3.1.19), cysteine desulfhydrase (CdsH; EC 4.4.1.15), and diaminopropionate ammonia-lyase (DapL; EC 4.3.1.15) generate 2AA from serine, cysteine and diaminopropionate, respectively. Once released, free 2AA may: i) covalently inactivate specific PLP-enzymes, potentially leading to growth defects, ii) be spontaneously quenched by solvent water, or iii) be deaminated by RidA as depicted in Figure 1 (3, 4, 6, 8, 12-17). In the absence of RidA *in vivo*, 2AA can inactivate PLP-dependent enzymes, including serine hydroxymethyltransferase (SHMT) (GlyA; EC 2.1.2.1), alanine racemases (Alr/DadX; EC 5.1.1.1), and transaminase B (IlvE; EC 2.6.1.42) (3, 6, 12, 14, 15). Transcriptomic analysis showed global changes in a *ridA* mutant of *S. enterica* and correctly predicted a motility defect, though the specific enzymatic target responsible for this effect was not found (18).

The biosynthetic threonine/serine dehydratase (IlvA) was the 2AA source responsible for the growth defects in multiple organisms that lacked the relevant RidA homolog, including

bacteria (*S. enterica* and *E. coli*), yeast (9, 11, 15 2001, 19) and plants (7, 20). As such, these defects were corrected by the addition of isoleucine, which allosterically inhibits IlvA to prevent 2AA production. Similarly, exogenous threonine prevents 2AA accumulation, likely by increasing flux to isoleucine which leads to allosteric inhibition of IlvA. In *S. enterica*, the growth defect caused by 2AA in a *ridA* mutant was reversed by exogenous glycine, which bypassed damage to the serine hydroxymethyl transferase (GlyA) (14, 21). In contrast, in an *E. coli ridA tdcF* double mutant, the 2AA-dependent growth defect was reversed by exogenous aspartate, or purines, but not glycine. Thus, while the mutant phenotypes that arise without RidA are expected to stem from 2AA inhibition of a PLP-dependent enzyme(s), the global metabolic consequences (i.e., growth phenotype) vary due to the unique metabolic architecture of each organism.

Pseudomonas aeruginosa PA01 encodes eight bioinformatically identified Rid proteins including, four RidA, one Rid1, and three Rid2 proteins (1, 22). The multiplicity of Rid proteins in *P. aeruginosa* suggests the proteins have distinct roles to adapt to changing conditions and/or act on substrates other than 2AA. For example, the Rid2 proteins (PA0814 and PA5083) increase the rate of FAD-dependent reactions *in vitro* (2). Additionally, PA5083 or *dguB*, is encoded in the *dguABC* operon and is positively regulated by exogenous D-Glu, however, its role in D-Glu utilization has not been demonstrated (23). Proteomic analyses linked RidA protein (PA5339) to early stage biofilm formation, while transcriptome analyses implicated RidA (PA3123) in biofilm formation and swarming, and showed Rid2 (PA5303) was induced in response to agmatine and putrescine (24-28). This study was initiated to probe Rid protein function and investigate the impact of 2AA on the *P. aeruginosa* metabolic network.

5.3 Materials and Methods

Bacterial strains, plasmids and media

The strains, plasmids and primers used in this study are listed in Table 1. *Pseudomonas aeruginosa* PAO1 mutants were obtained from the transposon mutant library collection and included the wild type strain MPAO1 (32). Transposon location was verified using a transposon-specific primer and a primer annealing to a flanking region of the chromosome. Derivatives of *S. enterica* serovar Typhimurium LT2 were used for complementation studies and *E. 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 (33), NCE or M9 with $MgSO_4$ (34) and trace minerals (35, 36) were used as a minimal medium base, as indicated. *P. aeruginosa* mutants were grown with 11 mM glucose. 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 as indicated, isoleucine, threonine, or glycine (1 mM), and serine (1 or 5 mM). L-arabinose (250 μM) was added to induce expression of genes inserted in relevant plasmids. Chemicals were purchased from MilliporeSigma (Sigma-Aldrich, St. Louis, MO).

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 replica printing to plates of the relevant medium. Alternatively, nutrients were spotted on soft agar overlays containing 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 following optical density at 650 nm at 37˚C with slow shaking speed. Overnight cultures of *S. enterica* or *P. aeruginosa* in biological triplicate were grown in rich medium at 37˚ C, pelleted and resuspended in an equal volume of sterile NaCl. Cell suspension was used to inoculate growth medium (2% inoculum) and growth was monitored for 24 hours. The resulting data were plotted using GraphPad Prism 7.0, generating curves in log10-format that display mean of three replicates and standard deviation of the mean. Specific growth (μ) were calculated according to the following equation: ln(X/X0)/T, where X is OD_{650}, X_0 is the starting OD_{650} of the exponential growth period monitored, and *T* is time in hours.

Molecular biology

A yeast (*Saccharomyces cerevisiae*) *in vivo* recombineering protocol was used to clone into the pMQ72 vector based on the Gibson cloning method and Shanks, *et al.* (29, 37-40). The plasmid pool from *S. cerevisiae* was isolated and electroporated into *E. coli*. Then 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.* (41) with a modified vector that contained the BspQI site (pCV1) (42). *P. aeruginosa* competent cells were prepared by standard methods, recovered in LB, and plated to selective medium at 37°C (43).

Protein purification

$_{PA}$RidA was purified from *E. coli* strain BL21AI harboring pDM1563 (pET28-*$_{PA}$ridA*) using the $_{SE}$RidA purification scheme described previously (44). 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 ÅKTA 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 uL) collected from the elution off the HisTrap HP column were analyzed via SDS-PAGE, and relevant fractions were identified by the presence of a ~ 13 kDa band. Fractions containing $_{PA}$RidA 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) using a PD-10 desalting column (GE Healthcare). Protein concentration was determined with the BCA protein assay kit (Pierce), and dilutions (0.125-2 μg) were loaded onto a 12.5% SDS-PAGE gel for protein purity analysis. $_{PA}$RidA 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 (4). Reaction mixtures (100 µL) contained Tris-HCl (100 mM, pH 8), NADH (250 µM), pyridoxal 5'-phosphate (30 µM), and pyruvate kinase/lactate dehydrogenase (5 Units). Purified cysteine desulfhydrase (CdsH) and the indicated Rid proteins were added at monomeric concentrations of 0.27 µM or 0.19 µM 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}).

Transaminase B activity assays

Fifty microliters of overnight NB cell culture was inoculated into 5 ml of minimal NCE medium containing 1 mM $MgSO_4$, trace minerals, 11 mM glucose and, when stated, 1 mM isoleucine or threonine. The cultures were incubated at 37°C with shaking until they reached full density. The cells were harvested by centrifugation and washed with NCE (1 mL). Cell pellets were frozen at -80°C until use. Cell pellets were resuspended in 1.0 ml of 50 mM potassium phosphate, pH 7.5. Cells were lysed using a Constant Systems Limited One Shot (United Kingdom) system by passing cells through the disrupter one time with the pressure set to 21,000 lb/in^2. The protein concentration was estimated using a bicinchoninic assay reagent kit (Pierce, Rockford, Ill.).

The transaminase B activity assay was an adaptation of previously described protocols (6, 45). A 50 µL aliquot of the whole-cell suspension was added to the reaction mixture and allowed to equilibrate at 37°C for 10 min. The reaction mixture contained 50 mM potassium phosphat(pH 7.5), 5 µL of 2 mM pyridoxal 5'-phosphate, 4 µL of 0.5 mM α-ketoglutarate, in a total volume of 200 µl. 20 µL of L-Isoleucine was added to start the reaction (final concentration of 20 mM). The reaction was allowed to proceed for 20 min at 37°C and stopped with 200 µl of 0.3% 2,4-dinitrophenyl-hydrazine. Hydrazone formation was allowed to proceed for 5 minutes at room temperature, prior to extraction with 1 ml of toluene and shaking for 2 minutes at 37˚C. The two phases were separated by centrifugation, and the aqueous (bottom) layer was removed by micropipette. The toluene layer was washed by adding 0.5 ml of 0.5 N HCl, vortexing, and separating the phases by centrifugation. A 0.8-ml aliquot of the toluene (top) layer was removed and mixed with 1 ml of 1.5 N, mixture was vigorously vortexed and centrifuged, then 200 µL was removed from the bottom layer. Absorbance was measured at A540 nm and results are reported in nmol/min/mg. The data presented as the mean of three biological replicates and error bars represent the standard error of the mean. Statistical significance ($P < 0.05$) was determined by conducting one-way analysis of variance (ANOVA) and Tukey's posttest using GraphPad Prism (version 7.0c).

Motility screens

Motility screens were performed by previously described methods (26, 46). M9 medium (20 mM NH_4Cl, 12 mM Na_2HPO_4, 22 mM KH_2PO_4, 1.0 mM NaCl, 1 mM $MgSO_4$) solidified with Bacto-agar (Difco), 0.3% for swimming or 0.7% for swarming motility, were prepared (36). Medium was autoclaved and cooled to 55 ˚C, trace minerals, 11mM glucose and, when applicable, 1mM isoleucine was added. 25 mL of medium was poured into Petri dishes in a single layer and

allowed to dry for 24 hours. Overnight cultures were grown in triplicate in LB plus antibiotic at 37 ºC, cultures were centrifuged and the pellet was resuspended in NaCl to an OD_{600} of 0.3. Ten µl of bacteria suspended was inoculated on each plate by gently stabbing into the soft agar, plates were then incubated at 37 ˚C for 24 hours. The diameter of each swimming halo was measured and reported in mm. The data represent the mean of three biological replicates and error bars represent the standard error of the mean. Statistical significance ($P < 0.00009$) was determined by unpaired Student's test (t test) using GraphPad Prism 7.0c.

Biofilm Formation Assays

Static biofilm assays were done as previously described (47, 48). Briefly, DM15943 and DM15944 were grown in biological triplicate overnight in LB with 100 µg/mL of gentamycin. Cultures were pelleted and resuspended in an equal volume of NaCl and a 1:100 dilution was made in to M63 minimal medium containing 11 mM glucose alone or with the addition of 1 mM isoleucine. 100 µL of cell dilution was used to inoculate a round-bottom 96-well microtiter plate in biological and technical triplicate. Plate was statically incubated for 20-24 hours at 37 ˚C, then rinsed with ddH_2O, dried, and stained with crystal violet. The stain was solubilized with ethanol, then the ethanol/stain was removed to flat-bottom 96-well microtiter plate and absorbance was read at 550nm. The data represent averages of technical replicates (within each biological replicate) and the mean of three biological replicates. The error bars represent standard errors of the mean from three biological replicates and one asterisks denote statistically significant ($P < 0.009$) variation between mutants determined by unpaired Student's test (t test) using GraphPad Prism 7.0c. M63 medium was used for these experiments to be consistent with the literature in the field, though the results are not expected to differ from those on NCE (47, 49-52).

5.4 Results

***Pseudomonas aeruginosa* mutants lacking PA5339 have a growth defect.** There are eight ORFs identified by the PubSEED database that encode Rid proteins in *Pseudomonas aeruginosa* PAO1 (Figure 2). Two of the ORFs defined as RidA proteins (PA3499, PA5392) do not have an active site Arg, making it unlikely they catalyze a deaminase reaction and these proteins were not included in this study. Five mutants of *P. aeruginosa*, each carrying an insertion in a single gene encoding a Rid protein, were obtained from the transposon mutant collection of the Manoil Laboratory (University of Washington). A mutant lacking PA5083, a Rid2, was not available. The correct insertion location for each mutant was validated by PCR using primers flanking the gene of interest. Initially, the mutants relevant to this study were screened for growth on a variety of media, both liquid and solid, including NCE minimal medium with glucose or glycerol as the sole carbon source and MOPS minimal medium with glucose or succinate as sole carbon source. On each of the media tested, growth of mutants with insertions in PA0814, PA1568, PA3123, or PA5303 was not detectably different from the parental strain (Figure S1). In contrast, the mutant with an insertion disrupting PA5339 had a significant growth defect as compared to wild type strain PAO1, in each minimal medium and NCE was used in further liquid growth analyses (Figure 3A). The growth defect was more severe on solid medium than in liquid though the reason for this was not pursued. The growth defect caused by inactivating PA5339 distinguished *P. aeruginosa* from *E. coli* and *S. enterica ridA* mutants in three ways. First, although disrupting *ridA* in *S. enterica* causes detectable biochemical effects, it has no significant effect on growth on minimal medium (12, 13, 15, 19). Second, the PA5339::Tn mutant is more sensitive to exogenous serine than a *S. enterica ridA* mutant (Figure S2). Third, in *E. coli*, both of the RidA homologs present had to be removed before any growth defect was detected (8). The growth defect

of *P. aeruginosa* mutants lacking only PA5339 showed that none of the other Rid homologs were functionally redundant under the conditions tested and, as such, PA5339 was renamed *ridA* (*$_{PA}$ridA* herein). This result could reflect distinct functional roles and/or differential regulation of the additional Rid subfamily members.

Nutritional requirements suggest conservation of Rid paradigm. Characterization of the general growth defect of PW9994 (*$_{PA}$ridA*::Tn) was guided by on our understanding of the RidA paradigm in *S. enterica.* Individual vitamins and amino acids were screened for growth stimulation on solid medium. In total these screens showed isoleucine, threonine, and to a lesser extent, glycine, proline and phenylalanine, stimulated growth of the *$_{PA}$ridA*::Tn mutant, where serine and cysteine were inhibitory. Growth of the wild type PAO1 strain was not affected by addition of these nutrients. For this reason, DM15943, *$_{PA}$ridA*::Tn/empty vector control, and DM15944 *$_{PA}$ridA*::Tn/pMQ72-*$_{PA}$ridA*, a complemented mutant were used as the isogenic pair for comparison throughout this study. The individual compounds that impacted the *$_{PA}$ridA*::Tn mutant growth most significantly were assessed in liquid growth curves. In minimal glucose medium (NCE), supplementation with isoleucine improved growth to wild type levels (Figure 3A), threonine had a similar effect, and glycine had little effect (S3). Isoleucine and threonine minimized the impact of a *ridA* mutation in *S. enterica,* by allosterically inhibiting IlvA to prevent 2AA synthesis (12, 13, 15). Similar to a *S. enterica ridA* mutant*,* serine (a precursor to 2AA) eliminated the growth of the *P. aeruginosa* mutant lacking PA5339. Addition of isoleucine improved, but did not fully restore growth of the *P. aeruginosa ridA* mutant to the level of the complemented *$_{PA}$ridA*::Tn mutant in the presence of serine (Figure 3B). In total, the behavior of the *P. aeruginosa $_{PA}$ridA*::Tn mutant could be explained in the framework of the RidA paradigm if 2AA generated from endogenous (on minimal medium) or exogenous serine damaged cellular

enzyme(s) and caused the growth defects observed. The restoration of growth by isoleucine suggests at least part of the 2AA was generated by serine/threonine dehydratase (IlvA) activity. *P. aeruginosa* encodes two IlvA proteins that each have both the catalytic and regulatory domains of the *S. enterica* protein, based on protein sequence comparison. Eliminating either one of these genes, *ilvA1* or *ilvA2* (PA0331 and PA1326), alone does not result in an isoleucine requirement, suggesting they are isozymes with redundant function in isoleucine biosynthesis. The failure of isoleucine to completely reverse the growth defect of the $_{PA}$*ridA*::Tn mutant on serine suggests, i) the serine/threonine dehydratase(s) are not completely inhibited by isoleucine, ii) more 2AA is produced by the IlvAs of *P. aeruginosa* than IlvA in *S. enterica,* or iii) there are other significant sources of 2AA in this organism.

Only PA5339 ($_{PA}$RidA) deaminated 2AA *in vivo* in the *P. aeruginosa* $_{PA}$*ridA*::Tn mutant. One explanation for the $_{PA}$*ridA*::Tn mutant growth defect despite encoding multiple Rid proteins, is that the homologs might not be expressed the under conditions tested. This explanation was not supported by complementation analyses in *P. aeruginosa*. The $_{PA}$*ridA*::Tn mutant was transformed with pMQ72 constructs harboring $_{PA}$*ridA*(pDM1533), PA3123/RidA (pDM1566), PA5083/Rid2 (pDM1534), or *S. enterica* RidA (pDM1568), under the control of an arabinose promoter or a no insert control (pMQ72) (29). Growth of the resulting mutants was monitored in minimal medium with 5mM serine. Plasmids expressing either $_{PA}$*ridA*, or $_{Se}$*ridA* fully restored growth to the level of the wild type PAO1 in the $_{PA}$*ridA*::Tn mutant without inducing expression by arabinose, and no other plasmid consistently improved growth (Figure S3).

The inability of PA5083, a Rid2, to complement the $_{PA}$*ridA*::Tn mutant was unexpected as this protein had previously been found to deaminate 2AA *in vitro* and complemented a *S. enterica ridA* mutant (2). A *S. enterica ridA* mutant (DM12920) was transformed with pBAD24 vectors encoding each of four Rid proteins from *P. aeruginosa*. The resulting mutants, along with controls DM14846 (pBAD24-$_{SE}$*ridA*) and DM14847 (pBAD24-empty vector), were assessed for the ability to grow in minimal glucose medium with 5mM serine. pDM1559 (pBAD24-$_{PA}$*ridA*) and pDM1439 (pBAD-$_{SE}$*ridA*) fully restored growth to the *ridA* mutant with or without added arabinose. The remaining *ridA* and *rid1,2,* pDM1561 (pBAD24-PA3123), pDM1464 (pBAD-PA0814), pDM1534 (pBAD-PA5083), restored growth of the *S. enterica ridA* mutant to varying degrees only when gene expression was induced by arabinose (Figure 4). In contrast, pDM1580 (pBAD24-PA5303), and pDM1587 (pBAD24-PA1568), failed to restore growth to an *S. enterica ridA* mutant (data not shown). The *S. enterica* complementation data indicated that in addition to PA5083, $_{PA}$RidA, PA3123, and PA0814 had 2AA deaminase activity *in vivo,* while, PA5303 and PA1568 did not. Although 2AA deaminases from each domain of life can be 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* (2). As with the other *Pseudomonas* Rid proteins, the inability of PA5303 and PA1568 to restore metabolic balance to a *S. enterica ridA* mutant might indicate protein stability or folding issues, which could account for the lack of complementation. It is also possible that PA5303 and PA1568 serve alternative roles in *P. aeruginosa* and may not substantially deaminate 2AA *in vivo*. Based on the metabolic defect generated solely by a insertion in $_{PA}$*ridA*, additional work focused on this protein and phenotypes of a $_{PA}$*ridA*::Tn mutant.

***P. aeruginosa* $_{PA}$RidA deaminates 2AA *in vitro*.** $_{PA}$RidA was purified and tested in an activity assay designed for $_{SE}$RidA, using cysteine desulfhydrase (CdsH) from *S. enterica* (4). The short half-life of 2AA requires it to be generated *in situ,* as the substrate for an enamine deaminase, in this case, $_{PA}$RidA or $_{SE}$RidA. CdsH converts cysteine to 2AA, which is then deaminated to form pyruvate. The rate of pyruvate formation is a combination of deamination by solvent water and a RidA protein (if present). Inclusion of $_{PA}$RidA in reaction mixtures, with multiple concentrations of cysteine (0.2-2 mM), increased the rate of pyruvate formation (Figure 5). These data showed that $_{PA}$RidA has 2AA deaminase activity *in vitro*, consistent with the ability of this protein to complement a *S. enterica ridA* mutant.

2AA accumulates and damages IlvE in a mutant lacking $_{PA}$*ridA*. The activity of the branched-chain amino acid aminotransaminase (transaminase B, IlvE EC 2.6.1.42) has been used as a proxy for *in vivo* 2AA damage both in *S. enterica* (6, 12, 13) and yeast (9). IlvE carries out the final step in the biosynthesis of isoleucine, leucine, and valine. The activity of IlvE decreases when RidA is absent, due to the accumulation of 2AA, which covalently damages this (and other) PLP-dependent enzymes *in vivo* (15). The *P. aeruginosa* genome encodes a single IlvE candidate, PA5013, with 48% identity to *S. enterica* IlvE. IlvE aminotransferase activity was assessed in mutants DM15943 ($_{PA}$*ridA*::Tn pMQ72-VOC) and DM15944 ($_{PA}$*ridA*::Tn pMQ72-$_{PA}$*ridA*) after growth in minimal glucose medium, or with the addition of isoleucine or threonine as indicated. The data in Figure 6 showed that an insertion in PA5339 (with empty vector) decreased IlvE activity ~ 2-fold compared to the mutant expressing $_{PA}$*ridA in trans*. In contrast, if isoleucine or threonine was added to the growth medium there was no significant difference in IlvE activity between the two mutants. These data suggest 2AA accumulates in a $_{PA}$*ridA*::Tn mutant and damages PLP-dependent enzymes. Further, the data are consistent with the *S. enterica* model

where the source of 2AA is a serine/threonine dehydratase, IlvA, that is regulated by addition of isoleucine (or indirectly by increasing threonine). Thus, 2AA accumulation is largely prevented in the uncomplemented *$_{PA}$ridA*::Tn mutant by addition of exogenous isoleucine or threonine.

2AA accumulation affects motility in a *$_{PA}$ridA*::Tn mutant. *S. enterica ridA* mutants have reduced motility (18), and mutants of *Campylobacter jejuni* (strain 11168) defective in a RidA homolog (Cje1388) are also impaired in motility and autoagglutination (30). The swimming motility of *$_{PA}$ridA*::Tn mutants DM15943 and DM15944 was assessed on minimal NCE and M9 glucose medium with 0.3% agar. The data showed that DM15943, a *$_{PA}$ridA*::Tn mutant carrying an empty vector, had a 3-fold decrease in swimming motility when compared to DM15944, a *$_{PA}$ridA*::Tn mutant carrying the *$_{PA}$ridA* complementation vector, on either medium (Figure 7). Motility of the PAO1 parent strain did not differ from DM15944 in any of the nutrient conditions tested (Figure S4). Critically, motility was restored by the addition of isoleucine to the medium, supporting the conclusion that reduced motility was due to an accumulation of 2AA. Unlike the relevant mutants in *S. enterica* and *C. jejuni*, the *$_{PA}$ridA*::Tn mutant of *P. aeruginosa* has a slight growth defect on minimal medium that prevents solid conclusions about the extent of the defect in motility caused by the lack of the RidA protein.

2AA accumulation leads to a defect in biofilm formation. A proteomics study found levels of PA5339 ($_{PA}$RidA) to be 5-fold higher at the onset of biofilm formation (i.e., during attachment) (25). Consistent with this result, a *$_{PA}$ridA*::Tn mutation had an effect on biofilm formation. The data show DM15943 had a 3-fold decrease in biofilm formation compared to DM15944 when mutants were grown in minimal M63 medium with 11 mM glucose as the sole carbon source (Figure 8). While M63 medium was used for consistency with the literature in the field, the stimulatory effect of exogenous isoleucine (1 mM), emphasized the conclusion that the

defect was due to 2AA accumulation. As with the motility defects above, it is difficult to distinguish the reduction in biofilm formation in a $_{PA}$*ridA*::Tn mutant from the minor growth defect in this mutant.

5.5 Discussion

The *P. aeruginosa* genome encodes eight Rid superfamily proteins, four of which have demonstrable 2AA deaminase activity *in vitro* and/or *in vivo*. Of the five mutants tested, each lacking one of the Rid homologs, only the mutant without $_{PA}$*ridA* (PW9994) had detectable mutant phenotypes in this study. The $_{PA}$*ridA*::Tn mutant displayed a nutritional phenotype, a biochemical defect, and was impaired in motility and biofilm formation. Each of these phenotypes were attributed to 2AA accumulation based on the exacerbation by serine and/or elimination by isoleucine. The growth defect present in the $_{PA}$*ridA*::Tn mutant complicates any conclusions about a defect in motility or biofilm formation. To confirm that 2AA accumulation causes a defect in either of these processes, like it does in at least *S. enterica* and *C. jejuni*, will require identification of a supplement that restores growth by overcoming the deleterious consequences of 2AA, rather than eliminating its production.

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) (3, 6, 16). *P. aeruginosa* carries two IlvA homologs encoded by genes *ilvA1* and *ilvA2* (PA0331 and PA1326). The presence of two IlvA homologues may account for the $_{PA}$*ridA*::Tn mutant increased sensitivity to serine as compared to *S. enterica* or *E. coli ridA* mutants (Figure S2). Both proteins in *P. aeruginosa* PAO1 have an allosteric regulatory domain, consistent with the addition of isoleucine preventing 2AA generation and minimizing the phenotypic defects caused by lack of $_{PA}$*ridA* (31).

Initial studies suggest *P. aeruginosa* differs from *S. enterica* in the 2AA paradigm. Isoleucine biosynthesis appears to be the major 2AA source in both organisms, but the data suggest there may be other significant source(s) in *P. aeruginosa*. While glycine restores growth to a *ridA* mutant in *S. enterica*, it has little effect on growth in the $_{PA}$*ridA*::Tn mutant when grown in minimal medium without serine (Figure S4). These data suggest the critical enzyme compromised in *P. aeruginosa* is not always serine hydroxymethyl transferase (GlyA), as it appears to be in *S. enterica*. These findings showed the *P. aeruginosa* system provides an opportunity to define a third PLP-dependent enzyme critical for growth. Despite the differences in 2AA stress between *S. enterica*, *E. coli*, and *P. aeruginosa,* these three organisms (and *C. jejuni*) appear to share a defect in motility related to 2AA accumulation. If the defect in motility in *P.* aeruginosa is confirmed to be independent of the growth defect, it will suggest a broad role for RidA in protecting a PLP-dependent enzyme involved in the regulatory and/or structural components of motility.

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Table 4.1 Strains and plasmids.

Strain ID	Genotype	Plasmid ID	Notes
S. enterica			
DM12920	*ridA1* ::Tn10d (Tc)	None	
DM14846	*ridA1* ::Tn10d (Tc)	pDM1439	pBAD24-*S.enterica-ridA*
DM14847	*ridA1* ::Tn10d (Tc)	pBAD24	pBAD24-empty vector
DM15406	*ridA1* ::Tn10d (Tc)	pDM1464	pBAD24-PA0814
DM15687	*ridA1* ::Tn10d (Tc)	pDM1515	pBAD24-PA5083
DM16214	*ridA1* ::Tn10d (Tc)	pDM1559	pBAD24-PA5339
DM16216	*ridA1* ::Tn10d (Tc)	pDM1561	pBAD24-PA3123
DM16390	*ridA1* ::Tn10d (Tc)	pDM1580	pBAD24- PA5303
DM16471	*ridA1* ::Tn10d (Tc)	pDM1587	pBAD24-PA1568
P. aeruginosa			
PW9994	PA5339::Tn	None	*PAridA* ::Tn
PW1611	PA0331::Tn	None	*ilvA1* ::Tn
PW3408	PA1326::Tn	None	*ilvA2* ::Tn
PW6252	PA3123::Tn	None	RidA (Supplemental figure 2)
PW2480	PA0814::Tn	None	Rid1 (Supplemental figure 2)
PW3822	PA1568::Tn	None	Rid2 (Supplemental figure 2)
PW9932	PA5303::Tn	None	Rid2 (Supplemental figure 2)
DM15943	*PAridA* ::Tn	pMQ72	Empty Vector
DM15944	*PAridA* ::Tn	pDM1533	pMQ72-PA5339
DM16327	*PAridA* ::Tn	pDM1566	pMQ72-PA3123 (Supplemental figure 2)
DM16328	*PAridA* ::Tn	pDM1568	pMQ72-ridA (LT2) (Supplemental figure 2)
DM16443	*PAridA* ::Tn	pDM1534	pMQ72-PA5083 (Supplemental figure 2)
DM15055	*E. coli* K12 ΔridA890 ΔtdcF14 araBAD::PBAD-ilvA219-cat	None	Supplemental figure 2
DM14931	*E. coli* K12 araBAD::PBAD-ilvA219-cat	None	Supplemental figure 2
DM9404	*S. enterica* wildtype	None	Supplemental figure 2
DM3480	*S. enterica ridA* ::MudJ	None	Supplemental figure 2

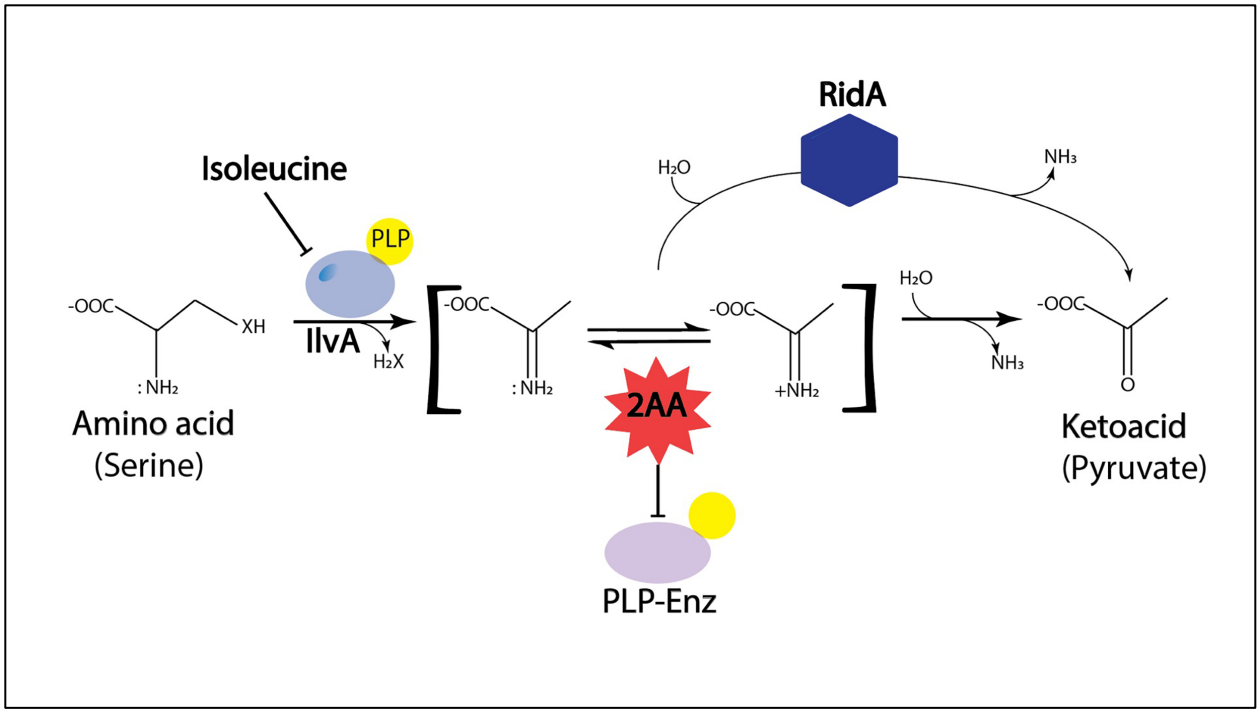
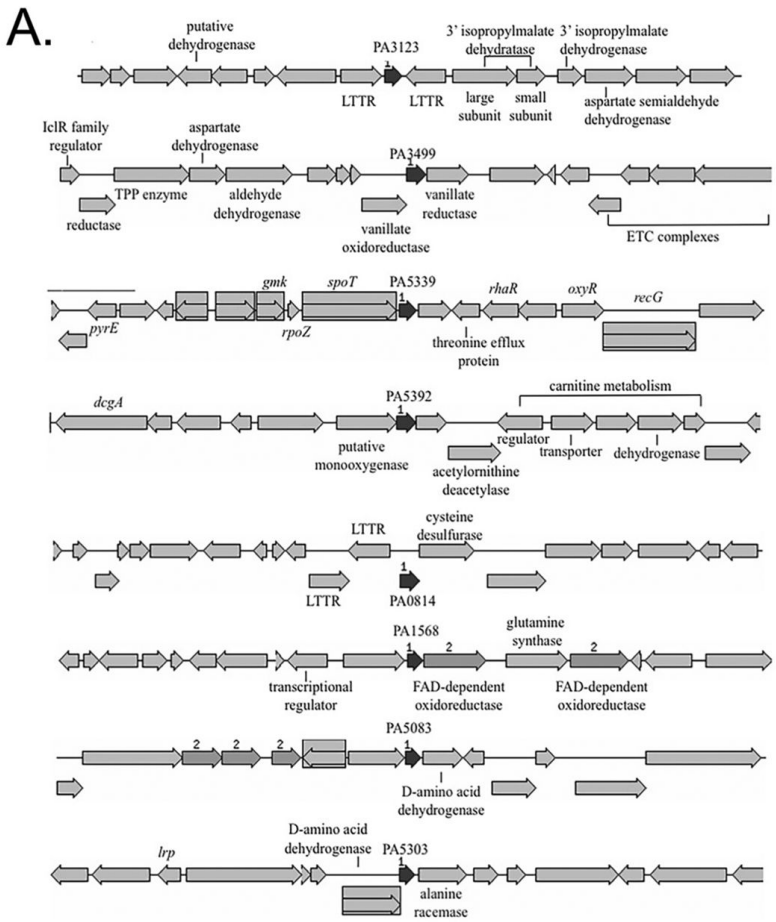


Figure 4.1: RidA paradigm in *S. enterica*. The framework for 2AA metabolism and its consequences as defined in *S. enterica* are depicted. 2-aminoacrylate (2AA, red) is generated from serine by PLP-dependent serine/threonine dehydratases (IlvA or TdcB) and released into the cellular milieu. 2AA is susceptible to spontaneous hydrolysis by H_2O, but in the absence of RidA, it persists long enough *in vivo* to damage to PLP-dependent target enzymes. Addition of isoleucine allosterically inhibits IlvA, decreasing the formation of 2AA by IlvA and preventing damage to metabolic target enzymes. This schematic shows how exogenous threonine can increase the synthesis of isoleucine which can then inhibit IlvA, reducing the accumulation of 2AA.



B. Locus Tag	Assigned Subfamily	Conserved Arg-105	2AA Deaminase Activity	
			in vivo	*in vitro*
PA3123	RidA	Yes	Yes (Figure 4)	No data
PA3499	RidA	No	Not expected	Not expected
PA5339	RidA	Yes	Yes (Figure 4)	Yes (Figure 5)
PA5392	RidA	No	Not expected	Not expected
PA0814	Rid1	Yes	Yes (Figure 4)	Yes (2)
PA1568	Rid2	Yes	No	No data
PA5083	Rid2	Yes	Yes (Figure 4)	Yes (2)
PA5303	Rid2	Yes	No	No data

Figure 4.2: Genetic organization of Rid superfamily members in *P. aeruginosa.* *P. aeruginosa* strain PA01 encodes eight Rid subfamily members. **(A)** In this schematic, taken from the PubSEED website (22), the Rid sub-family member is designated in black among neighboring genes in grey. **(B)** Characteristics of each Rid family member are listed using data from the listed

source, or the current study. *in vitro* 2AA deaminase activity indicates activity assays with purified protein. *in vivo* 2AA deaminase activity indicates the relevant protein complements the defects are caused by 2AA accumulation in a *ridA* mutant of *S. enterica*. NT= not tested.

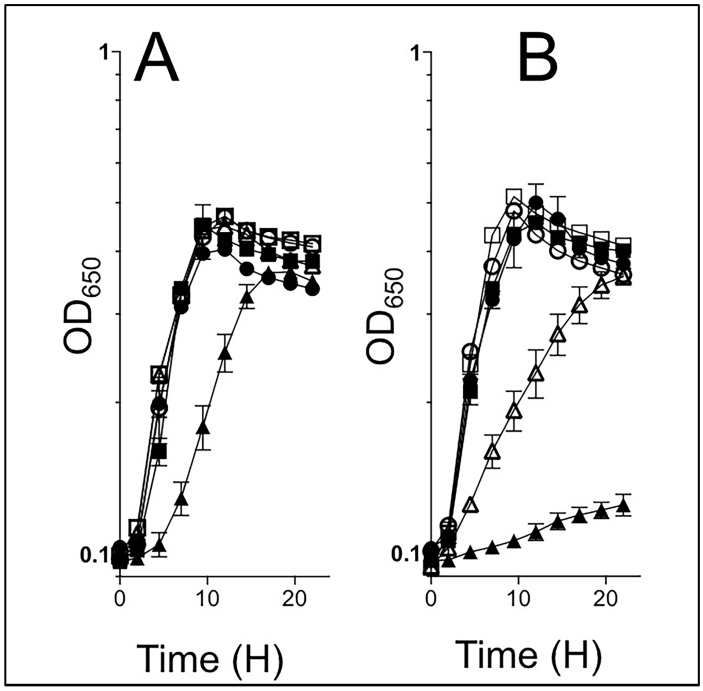


Figure 4.3: A *$_{PA}$ridA*::Tn mutant is sensitive to exogenous serine. Growth is shown for strains DM15943, *$_{PA}$ridA*::Tn/pEmpty vector (triangles), DM15944, *$_{PA}$ridA*::Tn/p*$_{PA}$ridA* (squares) and PAO1 (circles). Minimal medium with 11 mM glucose (closed symbols) and medium including 1 mM isoleucine (open symbols) were used. B) Growth is shown for strains DM15943, *$_{PA}$ridA*::Tn/pEmpty vector (triangles), DM15944, *$_{PA}$ridA*::Tn/p*$_{PA}$ridA* (squares) and PAO1 (circles). Growth was in minimal medium with 11 mM glucose and 5 mM serine (closed symbols), and medium with serine and 1 mM isoleucine (open symbols).

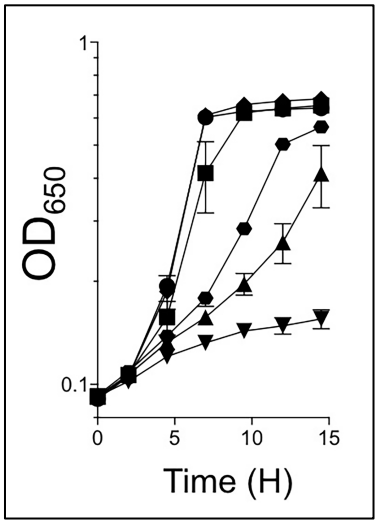


Figure 4.4: Rid homologs from *P. aeruginosa* complement a *S. enterica ridA* mutant. *S. enterica ridA* mutants carrying pBAD24 plasmids expressing the relevant genes were grown in minimal medium with glucose (11 mM), serine (5mM) and arabinose (0.2%). Strain DM14846 (circles) expressed *S. enterica ridA*, DM16214 (diamonds) expressed $_{PA}$*ridA*, DM15406 (squares) expressed PA0814, DM15687 (triangles) expressed PA5083 and DM16216 (hexagons) expressed PA3123. Control DM14847 (inverted triangles) carried an empty vector. DM14846 and DM16214 also grew in the absence of arabinose. The error bars represent standard errors of the mean from three biological replicates.

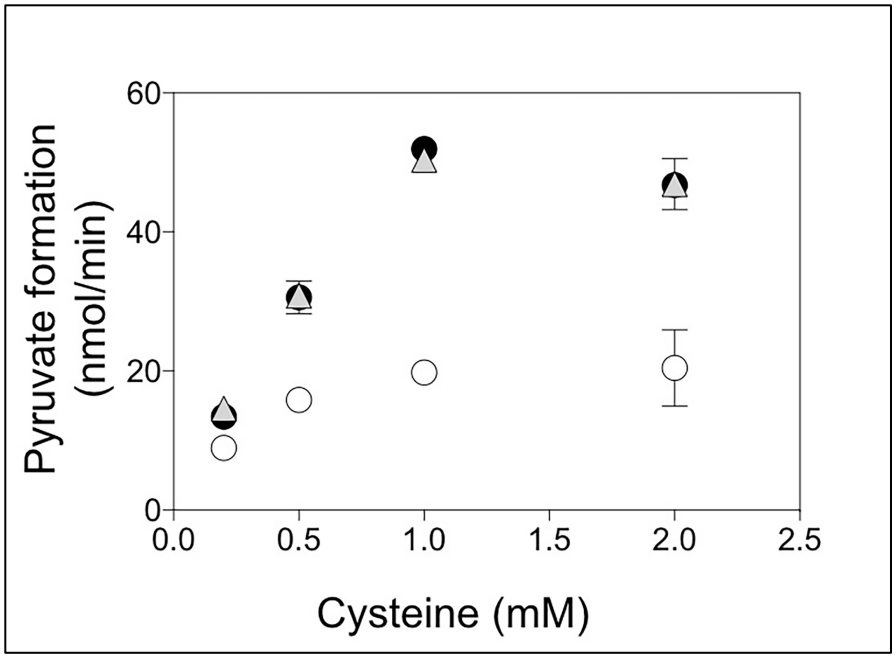


Figure 4.5: $_{PA}$RidA deaminates 2AA *in vitro*. *S. enterica* cysteine desulfhydrase (CdsH) was purified and used to generate 2AA from cysteine *in situ*. As previously described, coupled pyruvate formation and NADH oxidation are measured with and without addition of a Rid (either $_{SE}$RidA or $_{PA}$RidA) protein (4). NADH oxidation was used as a measurement of pyruvate formation. Then reaction rate was used to determine if $_{PA}$RidA deaminated 2AA *in vitro*, thus increasing the rate of the reaction. Assay mixtures (100 µL) contained TRIS-HCl (100 mM, pH8), NADH (250 µM), pyridoxal 5'-phosphate (30 µM), pyruvate kinase/lactate dehydrogenase (5 units), and purified CdsH (0.27 µM). Reactions contained $_{SE}$RidA (closed circles), $_{PA}$RidA(grey triangles) at 0.19 µM, or an equal volume buffer as a control (open circles). The reactions were initiated with L-cysteine addition to the indicated final concentrations and the reaction was monitored by absorbance at 340 nm for two minutes. 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 (ε = 6,200 M^{-1} cm^{-1}). Experiments were performed in triplicate and the mean was plotted with error bars representing the standard deviation.

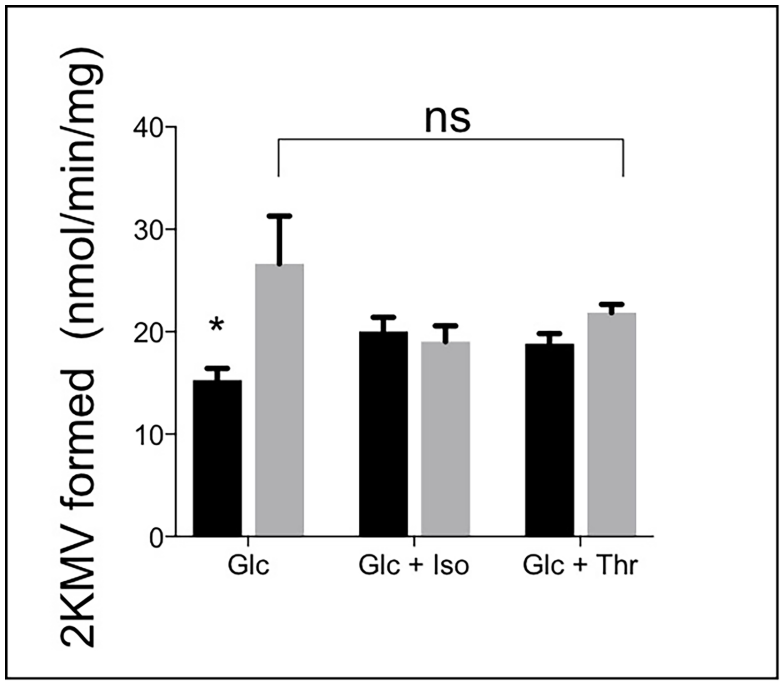


Figure 4.6: IlvE activity is decreased in a *$_{PA}$ridA::*Tn mutant. A *$_{PA}$ridA::*Tn mutant with pMQ72-empty vector (black) or pMQ72-*$_{PA}$ridA* (grey) were grown to full density in minimal glucose medium (Glc), with isoleucine (Glc + Iso), or with threonine (Glc + Thr). Transaminase b activity was assayed as described in the text. The error bars represent standard errors of the mean from three biological replicates and asterisks (*) denote statistically significant ($P < 0.05$) variation between strains.

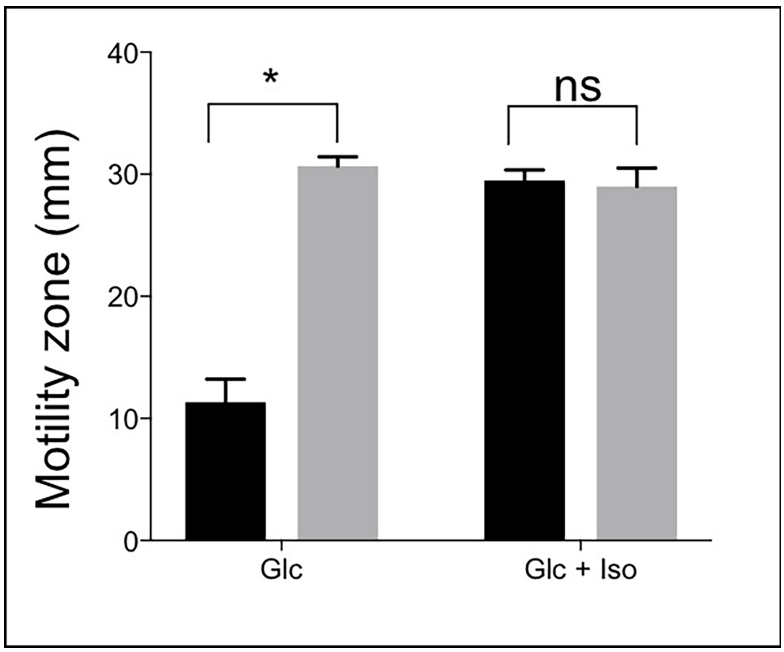


Figure 4.7: A *$_{PA}$ridA::*Tn mutant is defective in swimming motility. DM15943, a *$_{PA}$ridA::*Tn mutant with pMQ72-empty vector (black) and DM15944, a *$_{PA}$ridA::*Tn mutant with pMQ72-*$_{PA}$ridA* (gray) were inoculated on minimal motility plates, 0.3% agar, with glucose alone (Glc) or presence of 1 mM isoleucine (Glc + Iso). The error bars represent standard errors of the mean from three biological replicates and asterisks denote statistically significant ($P < 0.00009$) variation between strains.

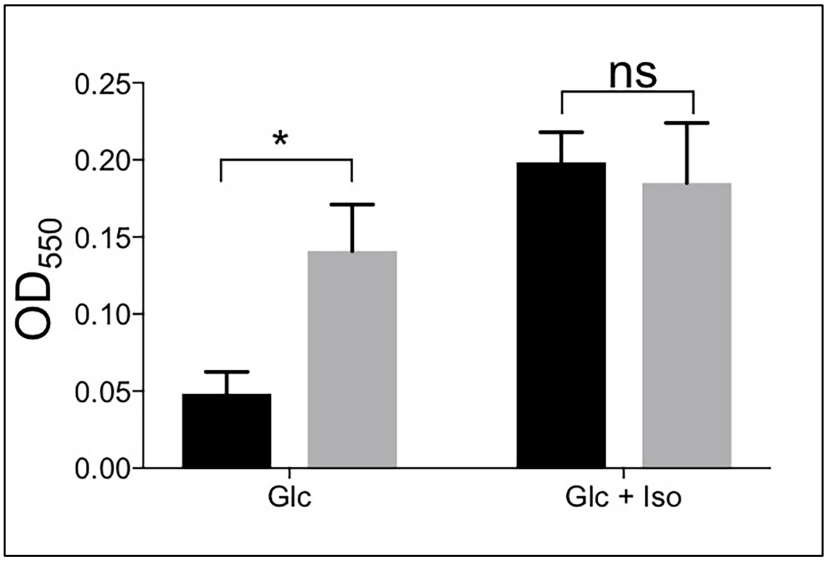
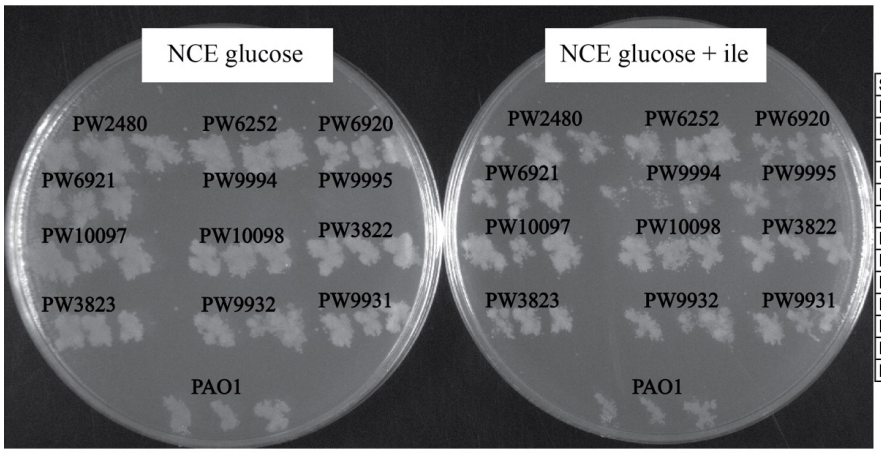


Figure 4.8.: A *$_{PA}$ridA::*Tn mutant is defective in biofilm formation. DM15943, a *$_{PA}$ridA::*Tn mutant with pMQ72-empty vector (black) and DM15944, a *$_{PA}$ridA::*Tn mutant with pMQ72-*$_{PA}$ridA* (gray) were grown in minimal M63 medium with 11 mM glucose as sole carbon source (Glc) or11 mM glucose with 1 mM isoleucine (Glc/Iso). The error bars represent standard errors of the mean from three biological replicates and one asterisks denote statistically significant ($P < 0.009$) variation between strains determined by an unpaired student's test (t test) performed using GraphPad Prism software version 7.0C).

Table S4.1: Primers used in this study.

Primer Name	Gene to amplify	Plasmid	Sequence
PR863	PA5339	NA	CGAGTACCGCATGGAAGTCT
PR864	PA5339	NA	CCCATGATCTCGTTGACCTT
PR857	PA3123	NA	GCCATGAGCACCAACTACCT
PR858	PA3123	NA	GTCGATATGAGCCAGGGTTG
PR851	PA0814	NA	GCTGTTGGAACCGTATTCGT
PR852	PA0814	NA	CGACAGAGGACGAGGAAGTC
PR869	PA1568	NA	GTATAGAGGGTGCCGTCCAG
PR870	PA1568	NA	GCTGTTCATGATGGGACTGA
PR871	PA5303	NA	CTCATCATCACGATCTTCGC
PR872	PA5303	NA	CCAGCACACTAACGAGCGTA
PR923	Sequencing	pMQ72 F	CCGCCAAAACAGCC
PR924	Sequencing	pMQ72 R	ACCCGTTTTTTTGGGCT
Universal	Sequencing	pBAD F	ATGCCATAGCATTTTTATCC
Universal	Sequencing	pBAD R	GATTTAATCTGTATCAGG
PR1037	LT2 ridA	pMQ72-*ridA* F	ccaagcttgcatgcctgcaggtcgactctagaggatccccTTAGCGACGAACAGCGA
PR1038	LT2 ridA	pMQ72-*ridA* R	tacccgttttttgggctagcgaattcgagctcggtacccCATTATGTGGTGCTGGCC
PR897	PA5339	pMQ72-*PAridA* F	TACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCGGTACCCGATTTCAGCAAGGA GTTCCC
PR898	PA5339	pMQ72-*PAridA* R	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCTTACTCGAGGACGA CGATG
PR1033	PA3123	pMQ72-PA3123 F	ccaagcttgcatgcctgcaggtcgactctagaggatccccCTCAGCCCTCGCCGACCCACGCC
PR1034	PA3123	pMQ72-PA3123 R	tacccgttttttgggctagcgaattcgagctcggtacccCGCAGTGCTACATGAATACGC
PR917	PA5083	pMQ72-dguB F	TACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCGGTACCCCCCTCGACTGGAGC ACTCC
PR918	PA5083	pMQ71-dguB R	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCtcaGCCCCTGGCCGC
PR978	pBAD24-PA5339 F	PA5339	NNGCTCTTCNTTCATGACCAAGACCGTTATCCAC
PR979	pBAD24-PA5339 R	PA5339	NNGCTCTTCNTTACTCGAGGACGACGATGG
PR976	pBAD24-PA3123 F	PA3123	NNGCTCTTCNTTCATGAGCGATGACATCCAGCGTTA
PR977	pBAD24-PA3123 R	PA3123	NNGCTCTTCNTTAGCCCTCGCCGACCCA
PR503	pBAD24-PA0814 F	PA0814	NNGCTCTTCNTTCATGCAGACATCCCCCGCTCCG
PR504	pBAD24-PA0814 R	PA0814	NNGCTCTTCNTTATCATGGCCGCACCGCCGC
PR1020	pBAD24-PA1568 F	PA1568	NNGCTCTTCNTTCATGCATATCGAACGTTTCGAAGT
PR1021	pBAD24-PA1568 R	PA1568	NNGCTCTTCNTTATCACTGCGGGCGGGC
PR1022	pBAD24-PA5303 F	PA5303	NNGCTCTTCNTTCATGCCCATCCAGCGCCAG
PR1023	pBAD24-PA5303 R	PA5303	NNGCTCTTCNTTATCAGGGCAGCGCAGCGAC
PR729	pBAD-PA5083 F	PA5083	NNGCTCTTCNTTCATGGAACCGACCCGTATCG
PR730	pBAD-PA5083 R	PA5083	NNGCTCTTCNTTATCAGCCCCTGGCCGCCAC



Strain ID	Genotype	Rid
PW2480	PA0814::Tn	Rid1
PW6252	PA3123::Tn	RidA
PW6920	PA3499::Tn	RidA
PW6921	PA3499::Tn	RidA
PW9994	PA5339::Tn	RidA
PW9995	PA5339::Tn	RidA
PW10097	PA5392::Tn	RidA
PW10098	PA5392::Tn	RidA
PW3822	PA1568::Tn	Rid2
PW3823	PA1568::Tn	Rid2
PW9932	PA5303::Tn	Rid2
PW9931	PA5303::Tn	Rid2
MPAO1	Wild type	

Figure S4.1: Only PA5339::Tn mutants have a growth defect under conditions tested. Mutants were streaked from frozen glycerol stocks to LB agar. Single colonies were patched in triplicate to LB agar and incubated four hours at 37˚C. The LB agar plate was replica printed to NCE/glucose (11 mM) and NCE/glucose/isoleucine (0.3 mM). Only PW9994 and PW9995 (PA5339:Tn) strains had a growth defect on NCE/glucose, which was corrected with the addition of isoleucine.

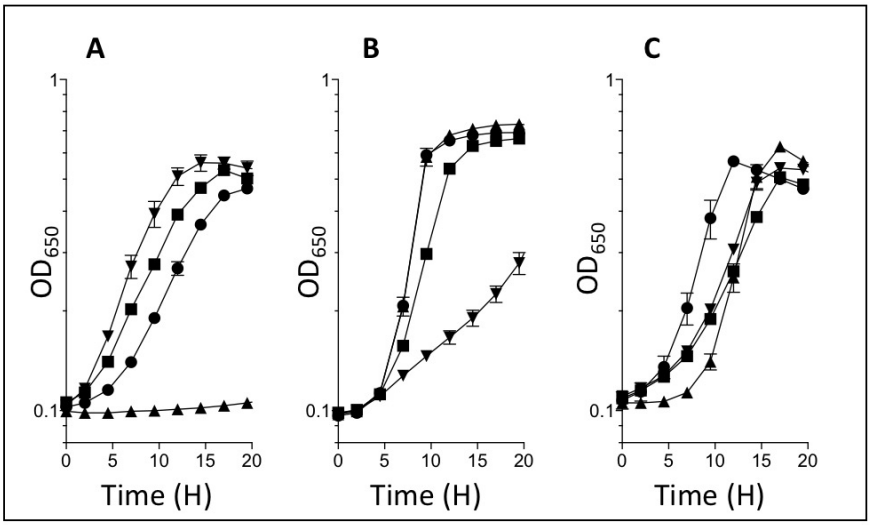


Figure S4.2: Sensitivity to serine differs among organisms lacking RidA. All organisms were grown in 20 mM glycerol +/- the addition of 3 mM serine and 0.2% arabinose (to induce

ilvA219 in E. coli chromosomal construct). The addition of 3 mM serine caused varying growth defects, suggesting 2AA sensitivity (from serine) differs in each organism. Historically, in minimal medium, 5 mM serine has been used to cause a growth defect in *S. enterica ridA* and *E. coli ilvA219* Δ*ridA* Δ*tdcF* mutants (1).

These data show, in minimal medium with glycerol, 3 mM serine completely inhibits growth of a *P. aeruginosa* $_{pa}$*ridA:Tn* mutant (A), partially inhibits growth of the *S. enterica ridA*::MudJ mutant (B), and does not inhibit growth of the *E. coli ilvA219* Δ*ridA* Δ*tdcF* mutant (C).

A) *P. aeruginosa* $_{pa}$*ridA*:Tn mutants: circles, DM15943 (pEmpty vector) glycerol only; squares, DM15944 (pPA5339) glycerol only; triangles, DM15943 glycerol/serine; inverted triangles, DM15944 glycerol/serine.

B) *S. enterica*: circle, wild type in glycerol; squares, *ridA*::MudJ in glycerol; triangles, wildtype in glycerol/serine; inverted triangles, *ridA*::MudJ in glycerol/serine.

C) *E. coli*: circles, DM14931 (*ilvA219*) in glycerol; squares, DM15055 (*ilvA219* Δ*ridA* Δ*tdcF*); triangles, DM14931in glycerol/serine; inverted triangles, DM15055 in glycerol/serine.

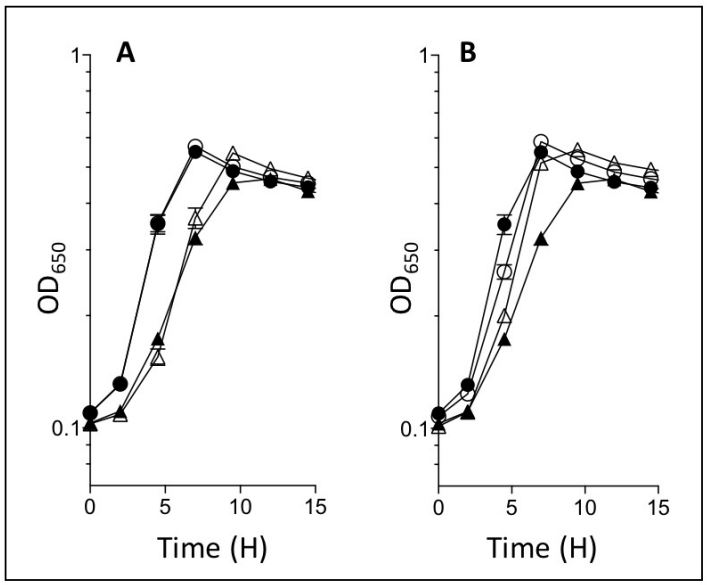


Figure S4.3: Growth of a *$_{pa}$ridA*::Tn mutant is improved by threonine but not glycine. A) Growth in minimal medium with 11 mM glucose (closed symbols), PAO1/WT (circles), PW9994 *$_{pa}$ridA*::Tn mutant (triangles), and with exogenous 1 mM glycine (open symbols). C) Growth in minimal medium with 11 mM glucose (closed symbols), PAO1/WT (circles), PW9994 *$_{pa}$ridA*::Tn mutant (triangles), and with exogenous 1 mM threonine (open symbols). The error bars represent standard errors of the mean from three biological replicates.

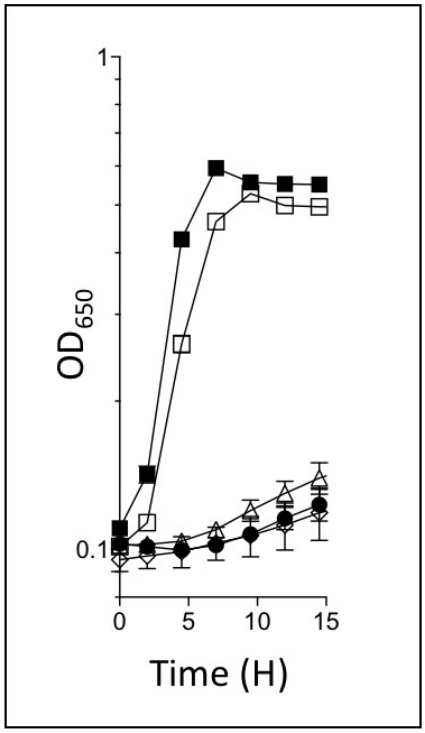


Figure S4.4: Growth of a *paridA*::Tn mutant is restored by *seridA* or *paridA in trans*. Expression of *seridA* or *paridA in trans*, DM16328 (squares) or DM15944 (open square), respectively, supported growth of the *paridA*::Tn mutant in minimal medium with 11 mM glucose and 5 mM serine. The empty vector control in DM15943 (circles), or expression of Rid family members, PA5083 in DM16443 (open diamonds), or PA3123 in DM16327 (open triangles) did not support growth of the *paridA*::Tn mutant. The error bars represent standard errors of the mean from three biological replicates.

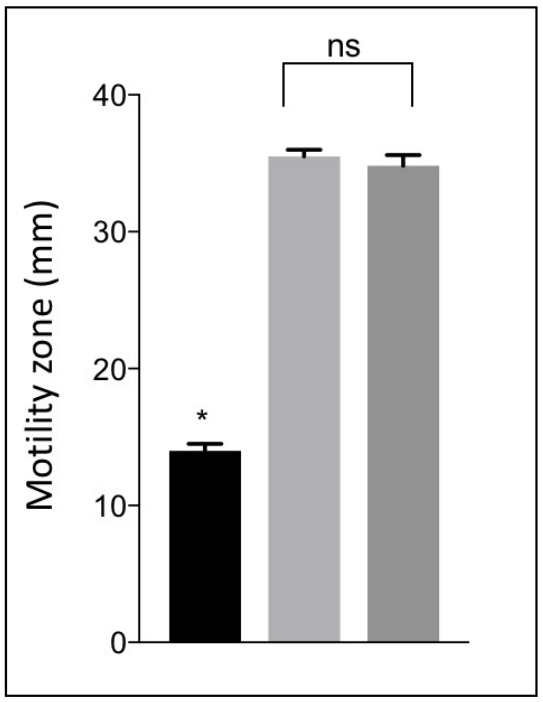


Figure S4.5: Motility of a DM15944, the complemented *$_{PA}$ridA*::Tn mutant are similar.

Diameter of motility was measured after 24 hours in minimal NCE glucose medium. There is a significant difference in motility diameter of DM15943 (black bar) as compared to DM15944 or PAO1. There is no significant difference between the motility of DM15944 and PAO1. Significance was determined by Students test and a P values <0.005.

CHAPTER 5

SUPPRESSOR MUTATIONS RESTORE GROWTH AND MOTILITY TO *RIDA* MUTANTS OF *PSEUDOMONAS AERUGINOSA*

Irons JL and Downs DM., to be submitted to the Journal of Bacteriology, January 1, 2020.

5.1 Abstract

Pseudomonas aeruginosa encodes eight members of the Rid superfamily of proteins (YjgF/YER057c/UK114). PA5339 is the RidA (reactive intermediate deaminase), and is required for full growth and motility in *P. aeruginosa.* The understanding of RidA integration into the metabolic network of *P. aeruginosa* is at an early stage with analysis largely guided by the well-established RidA paradigm from *Salmonella enterica. P. aeruginosa* lacking RidA had a severe motility defect in minimal glucose motility agar and, like *S. enterica,* growth and motility were abolished by exogenous serine and rescued by isoleucine. These data suggested the critical generator of the reactive metabolite, 2-aminoacrylate, in *ridA* mutants was a threonine/serine dehydratase. The critical targets of 2AA leading to growth and motility defects in *P. aeruginosa* were unclear. This study was initiated to define and further probe the metabolic integration of RidA in *P. aeruginosa* using suppressor analyses. Suppressor mutations that restored growth and/or motility in a *P. aeruginosa ridA* mutant were isolated and single-nucleotide polymorphisms were identified in *iscS* (cysteine desulfurase), *dapA* (dihydropicolinate synthase), *serA* (D-3-phosphpglycerate dehydrogenase), and *PA1559*. The potential significance of these loci in the enamine stress response of *P. aeruginosa* is discussed.

5.2 Introduction

The Rid (YjgF/YER057c/UK114) superfamily is found in all domains of life (1). The superfamily was divided into the eight subfamilies (RidA, and Rid1-7) based on phylogenetic grouping by the NCBI Conserved Domain Database (cd00448: YjgF_YER057c_UK114_family). Further sequence analysis placed the subfamilies into two groups 1) RidA and Rid1-3 which have a conserved active site residue, Arg105, and are predicted to have enamine/imine deaminase activity and 2) Rid4-7, which lack the critical arginine residue and are predicted to have distinct

functions (1). Most organisms encode at least one RidA homolog and the subfamilies, Rid1-7, exist only in prokaryotes. Members of the RidA subfamily from mammals, plants, yeast, and bacteria have been investigated over the past two decades.

The RidA (**r**eactive enamine/**i**mine **d**eaminase A) stress paradigm and conserved enamine/imine deaminase activity of RidA enzymes was elucidated primarily with biochemical and genetic studies in the model organism *S. enterica*. Conserved deaminase activity of RidA enzymes from human (UK114), goat (UK114), cucumber (ChrD), *Pyrococcus furiosus* (PF0668), *Bacillus subtilis* (YabJ), *Pseudomonas aeruginosa* (PA5339), *Campylobacter jejuni* (Cj1388), *Saccharomyces cerevisiae* (Mmf1p), *Yersinia pestis* (Y3551), and dust mite (Der F 34) has been demonstrated *in vitro* (2-7) (Martínez, in preparation). However, the integration of the deaminase activity into the specific metabolic network of the organism likely differs depending on the unique metabolic network.

In *S. enterica ridA* mutants, the reactive intermediate, 2-aminoacrylate (2AA) accumulated and damaged specific pyridoxal 5' phosphate (PLP)-dependent enzymes. 2AA generation and subsequent release from the enzyme active site has been linked to several enzymes in *S. enterica* including threonine/serine dehydratase (IlvA, EC 4.3.1.19), cysteine desulfhydrase (CdsH, EC 4.4.1.15), and diaminopropionate ammonia-lyase (DapL, EC 4.3.1.15) (2, 8, 9) which use serine, cysteine, or diaminopropionate, respectively. To date, the PLP-dependent enzymes known to be inactivated by 2AA include branched-chain amino acid aminotransferase (IlvE, EC 2.6.1.42), alanine racemases (Alr/DadX, EC 5.1.1.1), serine hydroxymethyltransferase (GlyA, EC 2.1.2.1), and aspartate aminotransferase (ApsC, EC 2.6.1.1) (10-14). Study of RidA integration into the unique metabolic network of distinct organisms has uncovered similarities and differences in the

PLP-dependent enzyme generators and/or targets of 2AA when comparing *S. enterica* to *Escherichia coli*, *P. aeruginosa*, *C. jejuni*, and *S. cerevisiae ridA* mutants (4-6, 14).

Motility defects of *ridA* mutants have been observed in multiple organisms including *S. enterica*, *P. aeruginosa*, and *C. jejuni*, suggesting the consequences of 2AA damage have far reaching consequences despite the distinct metabolic networks of each organism (5, 6, 14). The conserved deaminase activity of RidA proteins from all domains of life suggest, despite diverse phenotypes in each *ridA* mutant, the consequences are attributed to accumulation of 2AA and damage to PLP-dependent enzymes. Expansion of the RidA paradigm to various organisms provides an opportunity to gain insight into the unique metabolic network of each organism and to gain understanding of the broader impact of RidA enzymes on metabolism.

P. aeruginosa encodes eight members of the Rid superfamily with two RidA proteins (PA3123 and PA5339), one Rid1 (PA0814), three Rid2 proteins (PA1568, PA5303, and PA5083), and two Rid proteins without an active site Arg105 (PA3499 and PA5392) (5). Despite encoding multiple Rid family members, only a mutation in PA5339 led to metabolic defects under the conditions tested (5). In minimal medium with glucose, a *P. aeruginosa ridA* mutant had a growth and motility defect that were both corrected by exogenous isoleucine. Nutrient analysis suggested the generation and targets of 2AA accumulation in a *P. aeruginosa ridA* mutant may differ from *S. enterica ridA* mutants. When compared to an *S. enterica ridA* mutant, *P. aeruginosa ridA* mutants were sensitive to lower concentrations of serine and only slightly sensitive to cysteine despite encoding a cysteine desulfhydrase (PA1061). These data suggest generation of 2AA precedes largely through IlvA (*P. aeruginosa* encodes two IlvA, PA0331 and PA1326). The targets of 2AA damage to PLP-dependent enzymes remain undetermined but appear to differ from *S. enterica ridA* mutants. Unlike *S. enterica ridA* mutants, growth or motility of *P. aeruginosa ridA*

mutants is only improved by exogenous isoleucine, not by exogenous glycine or aspartate. The differences of *S. enterica* and *P. aeruginosa ridA* mutant phenotypes further underscore the metabolic distinctions between organisms and the influence on the phenotypic output of the system.

The cause of the motility defect resulting from lack of RidA, and whether it is direct or indirect, is unknown in any organism. To further probe the generators and targets of 2AA and causes of the motility defect in a *P. aeruginosa ridA* mutant, spontaneous mutants restoring motility were isolated and characterized. Of the suppressors identified, one recapitulated alleles that were previously shown to suppress a *S. enterica* ridA mutant and three were unique to *P. aeruginosa.*

5.3 Materials and Methods

Bacterial strains, media, and chemicals

The strains used in this study are listed in Table 5.1. *Pseudomonas aeruginosa* PAO1 wild type (MPAO1) and *ridA* (PW9994 *ridA*-F05::ISphoA/hah*)* were obtained from the transposon mutant library collection (15). Suppressor mutants were isolated from the *ridA* background and SNPs were determined by next-generation sequencing.

Lysogeny broth (LB) was used as a rich medium for *P. aeruginosa*. All *P. aeruginosa* mutants were grown at 37 ˚C. M9 (20 mM NH_4Cl, 12 mM Na_2HPO_4, 22 mM KH_2PO_4, 1.0 mM NaCl, 1 mM $MgSO_4$), trace minerals, and 11 mM glucose were used as a minimal medium base, as indicated (16-18). Supplements were added as indicated, isoleucine, valine, or leucine (1 mM), and serine (0.05 - 1 mM). Chemicals were purchased from MilliporeSigma (Sigma-Aldrich, St. Louis, MO).

Mutant isolation

Spontaneous suppressor mutants were isolated from the *ridA* background on solid minimal M9 medium with glucose and serine (0.5 mM) as follows, independent cultures (1 mL) of the *ridA* mutant were grown overnight in LB at 37˚C shaking, centrifuged, and resuspended in the same volume of saline. Cells (100 µL) were spread on solid medium with glucose and serine (0.5 mM) and incubated at 37 ˚C for 72 hours until spontaneous mutants arose. Suppressor mutants were streaked for isolation on solid minimal M9 medium with glucose and serine and were phenotypically characterized as discussed below.

Spontaneous motility suppressor mutants were isolated from minimal M9 motility agar (0.3%) with glucose as follows, independent cultures (1 mL) of the *ridA* mutant were grown overnight in LB shaking at 37 ˚C, centrifuged, and resuspended in saline (1 mL). The cell suspension (10 µL) was used to inoculate the center of the motility agar by gently piercing the top of the agar and expelling cells. Motility plates were incubated 37 ˚C for five days. After five days of incubation, asymmetrical outgrowths from the center motility halo were observed and a sterile toothpick was stabbed into cells furthest from the center inoculation point. The same toothpick was used to inoculate a second motility plate and to streak for isolated colonies on solid M9 minimal medium with glucose. Mutants that overcame the motility defect of the *ridA* mutant were further characterized.

Phenotypic analysis

Solid medium

Growth on solid and semi-solid medium was performed as previously described (5). Growth on solid medium was evaluated by patching strains to rich medium (LB), incubating plates overnight at room temperature, and replica printing to agar plates of the relevant medium.

Alternatively, 1.0 mL cultures were grown in LB overnight shaking at 37 ˚C, centrifuged, resuspended in an equal volume of saline, and 100 µL of the cell resuspension was embedded in 4.0 mL of soft agar. Nutrients were spotted on soft agar, plates were dried for 15 minutes at room temperature, and incubated overnight at 37˚C.

Motility medium

M9 motility medium with 0.3% Bacto-agar (Difco) was prepared as previously described (5, 18). M9 was filtered to sterilize. The water and agar mixture was autoclaved and cooled to 55 ˚C before 10x M9, trace minerals, glucose and, when applicable, 1mM isoleucine was added. 25 mL of medium was poured into Petri dishes in a single layer and allowed to dry for 24 hours at room temperature. Overnight cultures were grown in triplicate in LB at 37 ˚C, cultures were centrifuged, resuspended saline and set to an OD_{600} of 0.3. Bacterial suspension, 10 µL, was used to inoculate the center of each motility plate by gently piercing the top of the agar and expelling the cells. Plates were dried 15 minutes, then incubated at 37 ˚C for 24 hours or longer, as indicated. The diameter of each swimming halo was measured and reported in millimeter (mm) or as a percentage of the wild-type strain.

Liquid medium

Growth in liquid medium was assessed using a BioTek Elx808 microtiter plate reader following optical density at 650 nm at 37˚C with slow shaking speed as previously described (5). Overnight cultures of *P. aeruginosa* in biological triplicate were grown in LB medium at 37 ˚C, pelleted and resuspended in an equal volume of sterile saline. Cell suspension, 5 µL, was used to inoculate growth medium, 195 µL, and growth was monitored for 24 hours. Growth curves were plotted using GraphPad Prism (version 7.0).

Next-Generation sequencing and data analysis

Genomic DNA was extracted using the Monarch® genomic DNA purification kit (New England BioLabs). Libraries were constructed using Nextera™ DNA Flex library kit and analyzed using the iSeq 100 System (Illumina). Genomic sequence reads were realigned and mapped to the published PAO1 genome using Geneious software (version 10.1.2). High-frequency single-nucleotide polymorphisms (SNPs) were detected and respective impact on each coding sequence was predicted.

5.4 Results

Suppressors of a *P. aeruginosa ridA* mutant restore motility. Suppressor mutants with the ability to overcome growth and motility defects of the *P. aeruginosa ridA* mutant were isolated, phenotypically characterized, and the whole genomes were sequenced to identify single-nucleotide polymorphisms (SNPs). A previous study showed *P. aeruginosa ridA* mutants have a severe growth defect on minimal glucose agar and growth was completely abolished with the addition of serine (5). To isolate mutants that suppressed the serine sensitivity of the *ridA* mutant on solid medium, overnight cultures were spread on to M9 minimal glucose medium with serine (0.5 mM). Spontaneous mutants arose after 3-5 days. After 72 hours of incubation five independent spontaneous suppressor strains were chosen for characterization. Both growth and motility phenotypes were analyzed in these mutants. Two mutants that had distinct phenotypic profiles were sequenced to identify SNPs in the genome (Table 5.1, strains DM16723 and DM16724).

P. aeruginosa ridA mutants had a 3- to 4-fold reduction in motility compared to wildtype when grown in minimal glucose motility agar (0.3%) (5). After 5 days of incubation in motility agar, outgrowths from the center motility halo were observed on ~85% of plates. Sterile toothpicks were used to stab into motility outgrowths and isolate bacterial communities. Using this strategy,

30 independent mutants were screened and 20 exhibited improved motility. The 20 relevant mutants were further phenotypically characterized, and two mutants were chosen based on their different sensitivities to serine, valine, and leucine. Next-generation sequencing was used to identify SNPs in the genome (Table 5.1, strains DM17031 and DM17032).

Independently isolated spontaneous suppressor mutants from minimal medium with glucose and serine, or minimal motility agar with glucose were characterized for ability to suppress the growth and motility defects of a *P. aeruginosa ridA* mutant on/in solid, liquid, and motility media. Table 5.1 summarizes the four distinct lesions found in the strains that suppressed the *ridA* mutant phenotypes and Table 5.2 summarizes the motility and growth defects of each mutant strain. A single unique SNP was identified in each of four suppressor mutants using next-generation sequencing. The mutations were in *iscS* (DM16723), *PA1559* (DM16724), *serA* (DM17031), and *dapA* (DM17032) and will be discussed in detail below.

***P. aeruginosa ridA* mutants are sensitive to valine and leucine.** The phenotypic characteristics of *P. aeruginosa* wild type, *ridA*, and *ridA* suppressor mutants were analyzed in M9 minimal medium by soft agar overlays, replica printing, liquid growth curves, and motility assays. Valine and leucine sensitivity of suppressor mutants was first observed using M9 minimal medium by embedding cells in soft agar and assessing growth after spotting various nutrients on top agar. A *P. aeruginosa ridA* mutant had a severe growth defect in semi-solid or solid minimal media with glucose (5). In top agar overlays, no growth was observed on plates with the *P. aeruginosa ridA* mutant, the PAO1 wild type produced a robust lawn of growth, and suppressor mutants DM16723, DM16724, DM17031, and DM17032 had intermediate growth phenotypes (data not shown). Since the *P. aeruginosa ridA* mutant did not grow under these conditions, the intermediate growth of suppressor mutants led to more subtle observations that were previously missed. Suppressor

mutants had varying sensitivities to serine, valine and leucine, and isoleucine corrected the growth defects in/on solid or liquid medium. These phenotypes were quantified in liquid media (Figure 5.1 and Figure 5.3), and under these conditions, the *P. aeruginosa ridA* mutant also had sensitivities to valine and leucine, which were reversible with exogenous isoleucine.

***P. aeruginosa ridA* mutants display a weak temperature sensitivity.** The sensitivity of the *P. aeruginosa ridA* and suppressor mutants, DM16723 and DM16724, to serine, valine and leucine was assessed by replica plating. PAO1 (DM17029), the *P. aeruginosa ridA* mutant (DM17030), and suppressor mutants DM16723 and DM16724 were patched to LB and grown overnight at room temperature. After ~18 hours, the strains were replicate printed to M9 glucose minimal media plates with serine, valine or leucine (0.5 – 5.0 mM). The data are represented in Figure 5.2 where no growth is represented by light grey boxes, wild type growth is represented by dark grey, and intermediate growth (where cell density was less than wild type) is represented by white. Incubation at 42 ˚C improved growth of a *P. aeruginosa ridA* mutant on minimal glucose medium or minimal glucose with leucine or valine but not when serine was provided. DM16723 had no growth defect in minimal medium with glucose alone and was insensitive to exogenous serine, valine, or leucine at all temperatures. DM16724 displayed no growth defect on minimal media with glucose or with the addition of leucine, valine inhibited growth at 30˚C and growth improved as temperature increased, and growth was completely inhibited at all temperatures when serine was supplied. In total, these data showed that the growth defect of the *P. aeruginosa ridA* mutant could be overcome by increasing the temperature of incubation, DM16723 was insensitive to exogenous serine, and DM16724 was insensitive to leucine and valine, but not serine. It is unclear without further experimentation if the temperature sensitivity (or growth improvement) of the *P. aeruginosa ridA* mutant and suppressor mutants is simply a function of decreased (or

increased) growth rate or if *P. aeruginosa ridA* mutants are temperature sensitive due to metabolic perturbations caused by 2AA that are independent of growth the growth defect.

The *P. aeruginosa ridA* suppressor mutants had varying responses to exogenous serine when valine or leucine were supplied. Figure 5.3 shows the growth of *P. aeruginosa ridA*, and suppressor mutants DM16723, DM16724, DM17031, and DM17032 in liquid glucose media with serine (0.5 mM) and addition of valine, leucine or isoleucine. The *P. aeruginosa ridA* mutant had a growth defect in minimal glucose media with serine that was overcome by the addition of isoleucine (Figure 5.3A). Exogenous valine, not leucine further inhibited growth of the *P. aeruginosa ridA* mutant. All suppressor mutants were sensitive to serine, and valine exacerbated the growth defects to varying degrees. DM16723 was the least sensitive to serine, or serine with leucine or valine (Figure 5.3B). DM17031 was sensitive to serine under all growth conditions and growth was restored with isoleucine (Figure 5.3E). DM16724 and DM17032 had similar growth response to minimal glucose with serine and leucine or valine (Figure 5.3C and 5.3D). All of the growth defects caused by exogenous serine with valine or leucine were reversed when exogenous isoleucine is provided (data not shown). In total, nutritional analysis showed that all *P. aeruginosa ridA* suppressor mutants had reduced sensitivity to serine, leucine and/or valine, when compared to the parental *P. aeruginosa ridA* mutant, and isoleucine alleviated growth defects under all conditions tested. Interestingly, DM16723 was the best suppressor of the growth defect of a *P. aeruginosa ridA* mutant when serine was supplied, but was the worst suppressor of the motility defect. The significantly different phenotypes of the suppressor mutants further suggested the mode or degree of *P. aeruginosa ridA* suppression likely differed between mutants.

All of the isolated suppressor mutations improve motility in the *P. aeruginosa ridA* mutant. Each of the four suppressor mutants that was characterized improved growth and motility in minimal glucose motility agar (Figure 5.4 and Table 1.2). The *ridA* mutant had a 25% (4-fold) reduction in motility as compared to wild type. Despite being the least sensitive to exogenous serine, valine, and/or leucine, DM16723 displayed the lowest improvement in motility with motility 2.4-fold higher than the *ridA* mutant and 60% of wild type. DM16724 was the best suppressor of the *ridA* motility defect with 3.5-fold higher motility than the *ridA* mutant. Both of the suppressor mutants that were isolated from motility agar had motility that was ~80% of wild type and was 3-fold higher than the motility of the *ridA* mutant. Taken together these data demonstrate improved growth in the presence of serine (and/or valine and leucine) does not directly correlate with improvement in motility and suggests the suppressor mutants may be working through different modes of action to i) reduce 2AA accumulation, or ii) overcome damage (by 2AA) to a PLP-dependent enzyme.

A mutation in *iscS* restored growth and motility of a *P. aeruginosa ridA* mutant. Suppressor strain DM16723 had a mutation (A548C) that lead to the Q183P variant of IscS (PA3814), a cysteine desulfurase (EC 2.8.1.7). IscS has two domains, the small domain (residues 1–15 and 264–404) contains the critical active site cysteine (Cys328) and the large domain (residues 16–263) harbors the PLP cofactor, the cysteine substrate-binding pocket, and is responsible for dimerization (19). Previous structural studies of IscS indicate a Q183P variant may alter activity by disrupting normal coordination of bound-PLP in the IscS enzyme. It is unclear whether the mutation in *iscS* improved growth and motility of the *ridA* mutant by impacting 2AA accumulation or preventing 2AA enzyme damage. The possible modes of *ridA* suppression are addressed in the discussion.

An allele of hypothetical protein, PA1559 (*cprA*) led to improved growth and motility of the *ridA* mutant. A three amino acid deletion (ΔTCA188) lead to a I163S variant of PA1559, also named CprA (catatonic peptide resistance), and reduced serine sensitivity and improved motility in the *ridA* mutant. PA1559 is annotated as a pseudogene in PAO1 (Kegg.jp), is cited as a hypothetical protein in several transcriptomics studies and is said to play a role in PhoPQ-mediated polymyxin resistance in other strains of *P. aeruginosa* (20, 21). It is unclear how a mutation in PA1559 leads to improved growth and motility of a *ridA* mutant.

An allele of *serA* restored motility of a *P. aeruginosa ridA* mutant. A single base substitution in D-3-phosphpglycerate dehydrogenase (*serA*/PA0316 EC 1.1.1.95) that resulted in a R406H variant diminished the serine sensitivity and improved motility of a *ridA* mutant (DM17031). D-3-phosphoglycerate dehydrogenase (PGDH) uses NAD^+ as a cofactor in the first committed step in the phosphorylated serine biosynthesis pathway where PGDH converts phosphoglyceric acid to hydroxypyruvic acid phosphate (22, 23). PDGH is a tetrameric enzyme with three distinct domains including, the substrate binding domain (residues 7-108 and 296-317), the nucleotide binding domain (residues 108- 295), and the regulatory (serine) binding domain (residues 318-410) (24, 25). PDGH is allosterically inhibited by L-serine, the end product of the pathway, which binds to the regulatory domain (26). Upon binding the regulatory domain hinges into a closed conformation, making the active site inaccessibly (27). It is plausible that the suppressor mutation in *serA* leads to reduced flux towards serine and decreased 2AA accumulation in a *ridA*.

Alleles of *dapA* restored growth of the *ridA* mutant. The *P. aeruginosa ridA* suppressor mutant, DM17032, had a AA to GG substitution at bases 321 and 322 in the coding sequence that resulted in a N108G variant of dihydropicolinate synthase (*dapA*/PA1010 EC 4.3.3.7). In previous studies, six independent suppressor mutations mapped to the *dapA* locus in a *S. enterica ridA* mutant and eliminated the mutant growth defect in minimal medium with glucose and serine or in pyruvate alone (28). The variant DapA enzymes isolated from *S. enterica ridA* mutants had a 30-fold decrease in specific activity compared to the wild-type enzyme and were hypothesized to increase flux towards threonine and inhibit IlvA-mediated generation of 2AA (28). It is likely the *dapA* mutation in the *ridA* mutant background, similar to previously identified *dapA* mutations in the *S. enterica ridA* mutant, lead to increased flux to threonine and inhibition of 2AA accumulation.

5.5 Discussion

Phenotypic analysis of *ridA* suppressor mutants led to greater insights into the integration of RidA into the metabolism of *P. aeruginosa.* Nutritional analysis of *ridA* suppressor mutants supports a model where IlvA is the main generator of 2AA. Suppressor mutants of *ridA* were isolated for the ability to overcome 2AA stress associated with exogenous serine or the motility defect in minimal media. These mutants had an intermediate growth defect, between wild type (PAO1) and the *ridA* mutant, which allowed sensitivities to leucine and valine to be observed. The first step of isoleucine biosynthesis proceeds through IlvA using threonine or, alternatively, serine to produce pyruvate (with a 2AA intermediate). Both of the encoded IlvA (PA0331 and PA1326) enzymes in *P. aeruginosa* have the allosteric regulatory domain (determined by sequence analysis). The regulatory domain of IlvA allows allosteric inhibition by isoleucine and activation by binding to valine (29-34). Valine biosynthesis branches to feed into leucine biosynthesis and the first committed step of leucine biosynthesis proceeds through α-isopropylmalate synthase

(LeuA, EC 4.1.3.12). LeuA is inhibited by the end product of the pathway, leucine (35). These facts taken together led to a model where in a *ridA* mutant, exogenous leucine inhibited α-isopropylmalate synthase, increased metabolic flux to valine, and led to the activation of IlvA. Similarly, exogenous valine would directly activate IlvA. In either case, IlvA would use endogenous serine to produce 2AA which would accumulate and damage specific PLP-dependent enzymes resulting in the observed growth and motility defects. More experimental evidence will be required to determine if exogenous leucine and/or valine inhibit growth of the *ridA* mutant by increasing activity to one, or both, IlvA enzymes.

Nutrient and motility analysis of the *ridA* suppressor mutants suggested the mode or degree of suppression for each mutant differed. A single unique SNP was identified in each mutant including *iscS* (DM16723), *PA1559* (DM16724), *serA* (DM17031), and *dapA* (DM17031). The suppression by DM16724 and DM17031 will not be addressed further in this study. The hypotheses for the mode of suppression by IscS and DapA variants in the *ridA* mutant will be discussed below. In the context of the RidA paradigm, suppressor mutants likely change the metabolic network of *P. aeruginosa* in one of the following ways, directly or indirectly, the mutation may i) impact generation of 2AA and lead to a decrease in 2AA accumulation, ii) lead to an unusual molecule that quenches 2AA by interacting with the reactive intermediate, or iii) overcome 2AA damage.

The mutation in *P. aeruginosa dapA*, probably reduces generation of 2AA by increasing metabolic flux towards isoleucine. Isoleucine allosterically inhibits IlvA and 2AA accumulation. Dihydropicolinate synthase (*dapA*/PA1010 EC 4.3.3.7) uses aspartate 4-semialdehyde (ASA) as a substrate and participates in the synthesis of lysine derived from aspartate. Suppressor mutant isolation in the *S. enterica ridA* mutant resulted in six lesions in the *dapA* locus that corresponded

to decreased activity of DapA (28). Researchers hypothesized that decreased DapA activity led to increased endogenous threonine as metabolic flux to lysine was inhibited (28). Threonine led to inhibition of 2AA accumulation through inhibition of IlvA. Kinetic analysis of IlvA variants from *S. enterica* proposed when threonine is increased (endogenously or exogenously), flux to isoleucine would increase and lead to allosteric inhibition of IlvA by isoleucine (36). The suppressor mutation in *dapA* (DM17031) was isolated from minimal glucose motility agar. Growth and motility were improved with by the *dapA* allele. If the *dapA* allele suppresses the motility and growth defects by increased flux towards threonine and allosteric inhibition of IlvA by isoleucine, then it can be presumed IlvA is the main 2AA generator under motility and growth conditions. Further work is required to confirm that threonine accumulates endogenously in this mutant and IlvA activity is decreased.

A mutation in *iscS* (variant Q183P) suppressed the growth and motility defects of the *ridA* mutant by an unknown mechanism. IscS (also called NifS-like protein, cysteine desulfurase, EC 2.8.1.7) is a fold-type I PLP-dependent enzyme that mobilizes sulfur by desulfurization of cysteine to yield and IscS-bound persulfide and alanine (37). The Isc (iron-sulfur cluster) system involved in iron-sulfur cluster assembly and traffic of sulfur to several enzymes and tRNAs. As such, an *iscS* mutations have been shown to affect thiamine, biotin, NAD, isoleucine/valine, and molybdopterin biosynthesis; iron homeostasis; and tRNA activation (38-42). *E. coli iscS* mutants had decreased activities of several [4Fe-4S] containing enzymes including aconitase B, 6-phosphogluconate dehydratase, glutamate synthase, fumarase A, FNR, NADH dehydrogenase I, succinate dehydrogenase and as such, mutations in *iscS* could have far-reaching and diverse outcomes (43). *E. coli* and *S. enterica iscS* mutants have thiamine and nicotinic acid requirements (41, 43). *Saccharomyces cerevisiae* mitochondrial *ridA* (*mmf1*) mutants have a mitochondrial

DNA maintenance defect that can be attributed to a disruption in iron homeostasis (4). The *S. cerevisiae* retains a mitochondrial IscS homolog, Nfs1p that, if damaged by 2AA may lead to iron accumulation and mitochondrial DNA (mDNA) damage. The *nfs1* mutant of *S. cerevisiae* also has a decrease mitochondrial aconitase and succinate dehydrogenase activities (40, 44).

Based on the current status of this research, it is unclear how a mutation in *iscS* improves growth and motility of a *P. aeruginosa ridA* mutant. A study of structurally important amino acid residues of IscS from *E. coli* and the associated phenotypes of variant mutants found a change in residue 183 (Q183N) resulted in less tRNA modification, and crystal structures suggest Q183 is involved in the coordination of PLP in the enzyme active site (19). It is possible that the Q183P IscS variant has reduced or altered biochemical activity. Previous studies of *S. enterica ridA* suppressor mutants suggested inhibition of 2AA accumulation, quenching of 2AA by another molecule, or overcoming 2AA damage would each be feasible ways to improve defects caused by 2AA. To decrease generation of 2AA, an *iscS* mutant could lead to reduced flux to serine (the substrate used to generate 2AA by IlvA) or cysteine (the substrate used to generate 2AA by CdsH) or increased flux to isoleucine or threonine (both inhibit 2AA accumulation by serine) (8 2012). Suppressor mutants that increased flux to threonine or isoleucine have both improved growth in a *ridA* mutant of *S. enterica* (28). Alternatively, it is possible that the mutation in *iscS* could directly or indirectly lead to the formation of a product that quenches 2AA. This scenario has been hypothesized for multicopy suppressors of *S. enterica ridA* mutants that overcame 2AA stress by expression of *S. enterica metC* (PLP-dependent cystathionine *ß*-lyase, EC 4.4.1.8), *Methanococcus maripaludis* gene product MMP0739, or *E. coli ygeA* (putative PLP-independent aspartate/glutamate racemases) (45, 46). It was hypothesized that when MetC was overexpressed in a *ridA* mutant, a MetC-generated persulfide (thiocysteine) sequestered 2AA and prevented

accumulation and PLP-dependent enzyme damage by the reactive molecule (45). Similarly, *M. maripaludis* MMP0739 and *E. coli ygeA* were hypothesized to produce an intermediate in the racemization reaction that interacted with 2AA to form a stable product. It is possible that the variant $_{PA}$IscS is performing a moonlighting function that involves the enzyme-bound persulfide quenching 2AA *in vivo*. Or, the IscS variant could be producing a side product that interacts with 2AA independent of the enzyme. A *Lactobacillus bulgaricus* NifS-like protein expressed *in trans*, with 40% identity to $_{PA}$IscS, suppressed a leucine auxotrophy in an *ilvD ilvE* double mutant of *E. coli* and authors suggested their protein of interest had promiscuous aminotransferase activity (47). The third possible scenario for an *iscS* mutant mode of suppression is through bypassing 2AA stress directly or indirectly. This could be achieved by rerouting the metabolic network to produce the product of an enzyme damaged by 2AA. More work is needed to address the mode of suppression by the *iscS* allele in the *P. aeruginosa ridA* mutant.

The findings herein emphasize the role of IlvA in the accumulation of 2AA in the *P. aeruginosa ridA* mutant. We propose the suppressor mutation in *dapA* reduces 2AA accumulation by increasing metabolic flux to the allosteric inhibitor of IlvA, isoleucine. The mode of suppression by the allele of *iscS* is unknown, but we hypothesis either 1) the variant IscS enzyme produces a metabolite that quenched 2AA, similarly to suppression of a *S. enterica ridA* mutant by MetC or MMP0739, or 2) the mutation in *iscS* overcomes 2AA damage. Work to test these hypotheses and better understand the metabolic integration of RidA into the *P. aeruginosa* metabolic network is ongoing.

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Table 5.1. Strains used in this study.

Strain ID	Source	Chromosome
DM17029	Jacobs, 2003	MPA01 wild type (Manoil Lab)
DM17030	Jacobs, 2003	PW9994 *ridA* -F05::ISphoA/hah
DM16723	This study	*ridA* -F05::ISphoA/hah *iscS**
DM16724	This study	*ridA* -F05::ISphoA/hah *PA1559**
DM17031	This study	*ridA* -F05::ISphoA/hah *serA**
DM17032	This study	*ridA* -F05::ISphoA/hah *dapA**

Table 5.2. Summary of motility and growth of wildtype, *ridA*, and suppressor mutants.

Strain ID	Motility		Solid Media Growth		
	% WT Glc	% WT Glc/Ile	glc	glc/ser	glc/ile
DM17029	100%	100%	+	+	+
DM17030	25%	87%	-	-	+
DM16723	60%	83%	+	+	+
DM16724	87%	91%	+	-	+
DM17031	78%	94%	+	-	+
DM17032	78%	78%	+	-	+

Motility is represented by the percentage of wild type and was calculated by dividing the average diameter of motility of each mutant (from three biological triplicates) by the average diameter of motility of the wild type PAO1. Motility was measured in M9 minimal media with glucose alone or with addition of isoleucine. Growth of *ridA* and suppressor mutants was monitored on M9 minimal media with glucose alone or glucose and serine (1mM) or isoleucine. Wild type growth density is represented with a (+) and no growth is represented by (-) for each strain.

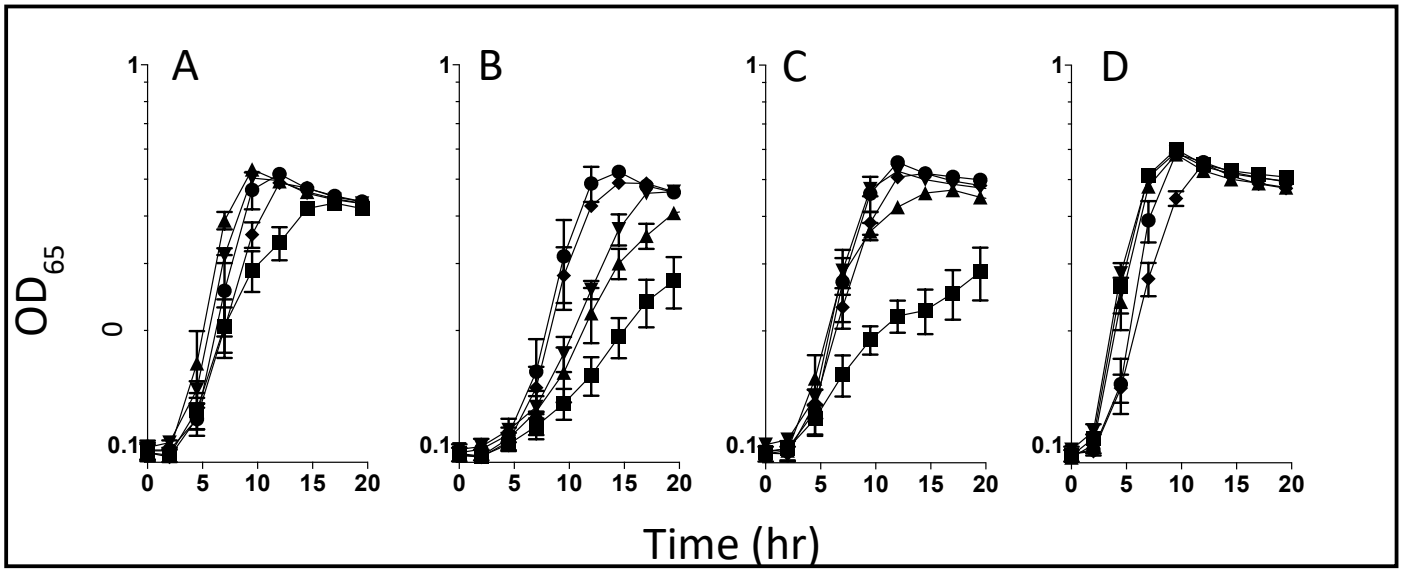

Figure 5.1: *ridA* suppressor mutants have varying degrees of sensitivity to valine and leucine. Mutants were grown in a 96-well plate format with M9 minimal medium with glucose alone (A), valine (B), leucine (C), or isoleucine (D). The *ridA* (DM17030) mutant was most sensitive to valine or leucine (square), DM16723 (inverted triangle) and DM16724 (triangle) were sensitive to valine and not leucine, DM17031 (circle) and DM17032 (diamond) were insensitive to valine or leucine.

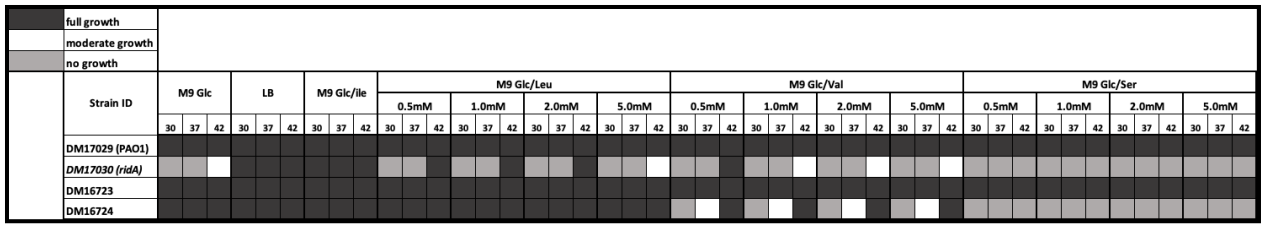

Figure 5.2: Temperature influences growth and sensitivity to leucine or valine. Wild type, PAO1 (DM17029), *ridA* mutant (DM17030), suppressor mutants DM16723 and DM16724 were replica plated to M9 minimal media with glucose (Glc) alone, or glucose with leucine, valine, or serine in concentrations ranging from 0.5 to 5.0 mM. Minimal plates were incubated at 30, 37, or 42 ˚C overnight and growth was scored full growth (dark grey) which equaled growth of the PAO1 strain, no growth (light grey), or moderate (white) which represents intermediate growth between PAO1 and no growth. As incubation temperature increased to 42 ˚C, growth inhibition of the *ridA* mutant became less apparent in glucose alone, or glucose with leucine or valine. DM16723 was insensitive to valine or leucine and DM16723 was less sensitive to valine as temperature increased.

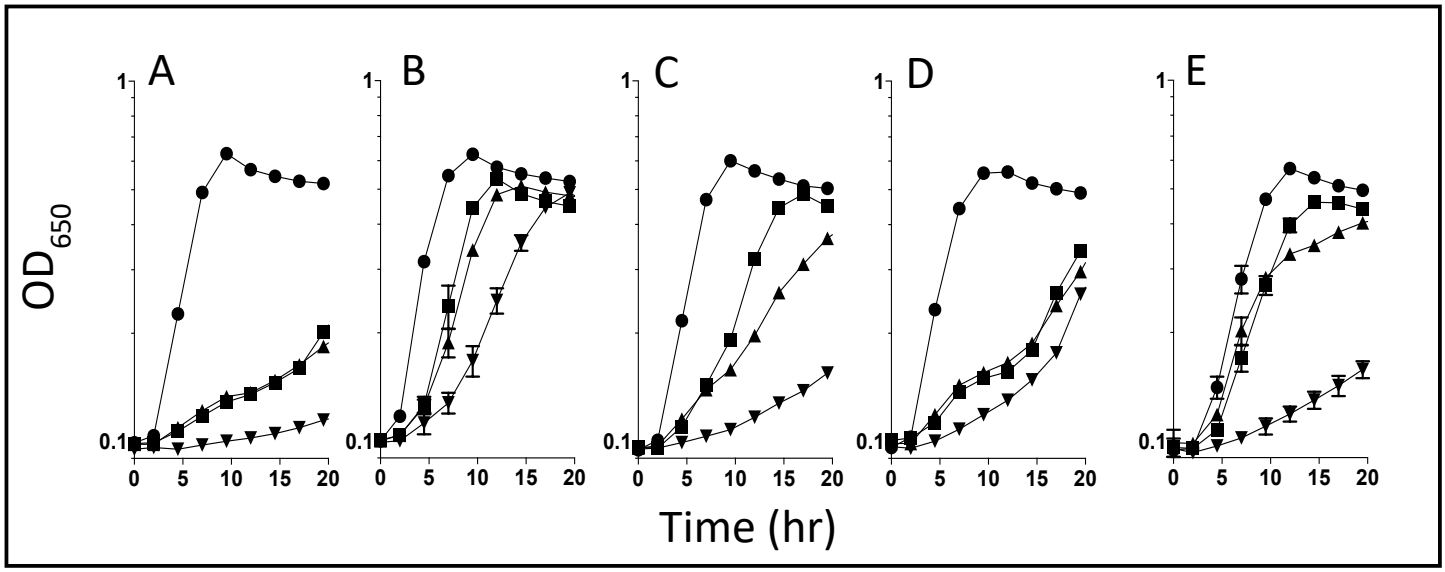

Figure 5.3: *ridA* suppressor mutants have varied sensitivities to valine and leucine when serine is present. Growth curves contain minimal M9 media with glucose and serine (0.5 mM) alone (squares) or with addition of isoleucine (circles), leucine (triangles), or valine (inverted triangles). The *ridA* mutant (A) was sensitive to serine alone and growth was further inhibited when valine was supplied. DM16723 was the least sensitive to serine or serine with leucine or valine (B), DM16724 was sensitive to serine and valine (C), DM17031 was sensitive to serine alone or with leucine or valine (D), DM17032 showed little sensitivity to serine alone or serine and leucine, but was sensitive to valine and serine (E).

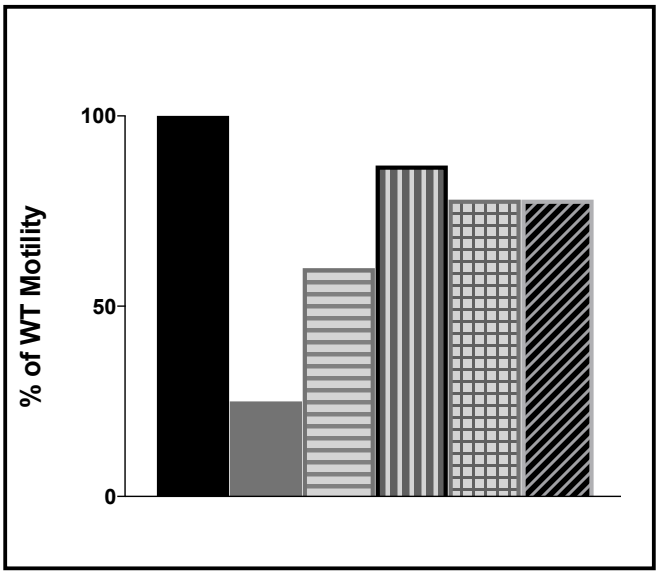

Figure 5.4: All *ridA* suppressor mutants improve motility. Motility of all strains was determined by motility assays performed in biological triplicate. The wild type (PAO1) motility was set to equal 100 percent and each mutant motility was determined as a percentage of wild-type motility. Motility of PAO1 (black), *ridA* mutant (gray), DM16723 (horizontal stripe), DM16724 (vertical stripe), DM17031 (checkered), and DM17032 (diagonal stripe) were compared in M9 minimal glucose motility agar (0.3%). The *ridA* mutant has a 4-fold decrease in motility when compared to PAO1. All suppressor mutants improve motility, but none equal PAO1.

CHAPTER 6

CONCLUSIONS

6.1 The metabolic network influences 2-aminoacrylate stress.

The work presented in chapter 3, 4, and 5 demonstrated the physiological impact of 2-aminoacryclate (2AA) accumulation in the different metabolic contexts of *Salmonella enterica, Pseudomonas aeruginosa,* and *Campylobacter jejuni.* RidA (reactive intermediate deaminase A) enzymes are found in all domains of life and have been implicated in a variety of cellular processes. *S. enterica* was used as a model system to establish the physiological role of RidA and the conserved biochemical activity of RidA enzymes was demonstrated using purified proteins from diverse organisms that spanned the domains (1). In *S. enterica ridA* mutants, 2AA generation proceeds through pyridoxal 5' phosphate (PLP)-dependent enzymes including biosynthetic serine/threonine dehydratase (IlvA, EC 4.3.1.19), cysteine desulfhydrase (CdsH EC 2.5.1.47), or diaminopropionate lyase (DapL, EC 4.3.1.15) (1-4). Once released from the enzyme activity site, 2AA damages other specific PLP-dependent enzymes and, to-date, is known to damage branched-chain aminotransferase (IlvE, EC 2.6.1.42), glycine hydroxymethyltransferase (GlyA, EC 2.1.2.1), alanine racemase (Alr/DadX, EC 5.1.1.1), and aspartate aminotransferase (AspC, EC 2.6.1.1) (5-8). As RidA enzymes are studied in the metabolic context of different organisms, the unique metabolic architecture of each organism is expected to influence the framework of the established paradigm. Specifically, depending on the metabolic integration of RidA, the major enzyme generator and critical enzyme targets of 2AA may change and these differences generate

fundamental insights into the unique metabolic network of the organism. This work aimed to expand understand of the RidA paradigm in diverse bacterial species and to gain understanding the metabolic networks of *P. aeruginosa,* and *C. jejuni.*

6.2 2-aminoacrylate stress in *Campylobacter jejuni* impacts flagella.

Chapter 3 described the phenotypes of a *C. jejuni ridA* mutant. *C. jejuni* encodes one $_{Cj}$RidA (Cj1388) and one Rid2 (Cj0327) enzyme, yet, a mutation in $_{Cj}$RidA alone was sufficient to elicit a defect in flagellar biosynthesis, structure, or function. The *C. jejuni* $_{cj}$*rid*A mutant had a severe motility defect and transmission electron microscopy suggested a distinct difference between the flagellum number and/or structure when RidA was absent. Both Rid proteins, $_{Cj}$RidA and Cj0327, were confirmed to share the conserved deaminase activity of RidA enzymes *in vitro* and *in vivo*. $_{Cj}$RidA and Cj0327 expressed *in trans* complemented a *S. enterica ridA* mutant growth defect. The motility defect of *C. jejuni* $_{cj}$*rid*A was chromosomally complemented by $_{cj}$*rid*A ($_{cj}$*rid*A::kan cj0046::$_{cj}$*rid*A). This work began to demonstrate the unique impact of 2AA accumulation on *C. jejuni.* The 2AA generators and targets of a *C. jejuni ridA* remain undetermined, however, under the conditions tested, it was demonstrated that i) IlvA was the biosynthetic threonine dehydratase and ii) IlvA was not responsible for 2AA accumulation that lead to flagellar defects in rich media. A comparison of *ridA* mutants of *S. enterica* and *C. jejuni* offer a clear example of how differences in metabolic network structure impact generation of and cellular response to 2AA.

6.3 *Pseudomonas aeruginosa* experiences 2-aminoacrylate stress.

Chapter 4 described work to expand the RidA paradigm to *P. aeruginosa* and to understand how the unique metabolic network structure influences 2AA generation and damage. *P. aeruginosa* encodes eight Rid proteins, yet, only an insertion in *PA5339* led to a detectable

phenotype under the conditions tested. This work confirmed PA5339 was a bona fide RidA enzyme. $_{PA}$RidA was confirmed to be a 2AA deaminase by *in vitro* assays where 2AA was generated from purified *S. enterica* cysteine desulfhydrase and *in vivo* by complementation in both a *P. aeruginosa ridA* mutant and a *S. enterica ridA* mutant. Like a *S. enterica ridA* mutant, the *P. aeruginosa ridA* mutant had a growth and motility defect that were alleviated by exogenous isoleucine These data suggest generation of 2AA by IlvA is conserved in *P. aeruginosa*. Unlike *S. enterica*, the critically damaged enzyme in *P. aeuginosa ridA* mutants was not GlyA which supports the conclusion that the diverse metabolic structure of each organism influences generation and/or response to 2AA accumulation.

6.4 *Pseudomonas aeruginosa* overcomes 2-aminoacrylate stress with various mutations.

Chapter 5 described work to probe the integration of RidA into the metabolic network of *P. aeruginosa*. Suppressor mutants were isolated from the *P. aeruginosa ridA* mutant by selection on minimal medium with glucose and serine or from minimal glucose motility agar. The suppressor mutants were phenotypically characterized, and a subset of the isolated mutants was sequenced using Next-generation sequencing. A unique single nucleotide polymorphism (SNP) was identified in each mutant and corresponded to a mutation in *iscS*, *PA5339*, *serA*, or *dapA*. Nutritional screens of the $_{pa}$*ridA* and suppressor mutants uncovered valine and leucine sensitivities. These data support the assumption that IlvA is the main generator of 2AA in *P. aeruginosa.* Work is on-going to understand the mode of suppression in each mutant.

6.5 Future directions

Work to understand RidA integration into the metabolic networks of *C. jejuni* and *P. aeruginosa* is in the early stages. Many questions remain to understand i) how RidA influences the metabolism of each organism, ii) how the reactive metabolite (2AA) can perturb complex and diverse systems, and iii) what are the fundamental metabolic difference between these organisms?

C. jejuni RidA ($_{Cj}$RidA/Cj1388) was cited in several -omics studies that linked $_{Cj}$RidA to acid adaptation, host colonization, antibiotic resistance, and environmental stress (9-15). Two studies showed that *C. jejuni ridA* was differentially regulated in response to heme acquisition, making it the first RidA known to be regulated in response to environmental conditions (16, 17). Directed studies showed *C. jejuni ridA* mutants had attenuated virulence in a *Galleria mellonella* infection model and increased sensitivity to H_2O_2, but since $_{cj}$*ridA* was misannotated, those phenotypes were not examined in the context of 2AA accumulation and enzyme damage (13, 16). The work in chapter 3 began to address the phenotypes of *C. jejuni* lacking RidA in the context of the well-established RidA paradigm. Though, questions remain to better understand how $_{Cj}$RidA is integrated into the metabolic network of *C. jejuni*. What are the generators and critical targets of 2AA in *C. jejuni ridA* mutants? Are the flagellar defects of the *C. jejuni ridA* mutant caused by direct 2AA damage to a specific enzyme or are there indirect and far-reaching impacts of 2AA? Is the reduced virulence of the $_{cj}$*ridA* mutant a direct result of 2AA accumulation or an indirect effect of flagellar defects? Is the reported H_2O_2 sensitivity of the $_{cj}$*ridA* mutant repeatable and is it a direct consequence of 2AA accumulation? Under what physiological conditions would repressing transcription of $_{cj}$*ridA* be beneficial and why? The previous observations of $_{cj}$*ridA* regulation and the varying the phenotypes of $_{cj}$*ridA* mutants reported in the literature offer opportunities to

generate fundamental insights in the metabolism of *C. jejuni* and to learn about the physiological role of RidA in a unique metabolic network.

P. aeruginosa was used to expand understanding of the metabolic role of RidA in another diverse bacterial species. The early studies of the *P. aeruginosa ridA* mutant demonstrated the unique integration of RidA into this metabolic network. *P. aeruginosa* is an opportunistic pathogen that can infect the human lung, skin wounds, and intestines. *P. aeruginosa* has several redundant proteins including eight Rid enzymes, two serine/threonine dehydratases (2AA generator), three glycine hydroxymethyltransferases (2AA target) among others. Given the unique metabolic network, it is not surprising that the RidA paradigm in *P. aeruginosa* would shift, from what is known in *S. enterica,* and uncover new connections. Several questions remain to understand RidA in metabolic context of *P. aeruginosa*. How do the 2AA generators and targets differ in *P. aeruginosa*? Does a mutation in $_{pa}$*ridA* lead to decreased virulence in a *Drosophila melanogaster* infection model? Is the observed cold sensitivity of a $_{pa}$*ridA* mutant independent of the general growth defect? Can the physiological function of other Rid proteins be observed phenotypically or biochemically?

Generation of and damage to PLP-dependent enzymes by 2AA in a *S. enterica ridA* mutant differs from a *P. aeruginosa ridA* mutant. *P. aeruginosa* encodes two known generators of 2AA, redundant serine/threonine dehydratases (*PA0331* and *PA1326*) and a cysteine desulfhydrase (*PA1061*). The *P. aeruginosa ridA* mutant is slightly sensitive to exogenous cysteine and is sensitive to even low concentrations of serine. The $_{pa}$*ridA* mutant has a growth and motility defect in minimal medium with glucose alone, and both defects are alleviated with exogenous isoleucine (18). Taken together, these data suggest one or both IlvAs encoded by *P. aeruginosa* are the main generators of 2AA in a *ridA* mutant and endogenous serine is sufficient to elicit metabolic defects.

A genetic and biochemical approach should be used to further probe the role of IlvA enzymes, PA0331 and PA1326, in 2AA generation. To determine if one (or both) of the IlvA enzymes is the critical 2AA generator, single and double mutants of *PA0331* (*ilva1*) and *PA1326* (*ilvA2*) could be constructed in a $_{pa}$*ridA* background and growth and motility examined. If growth and motility are restored in the single, *ilvA1* or *ilvA2*, or double mutant background, it would indicate one or both of the IlvA enzymes were the main generators of 2AA in *P. aeruginosa*.

The critical enzyme targets of 2AA damage in the *P. aeruginosa ridA* mutant are currently unknown. The $_{pa}$*ridA* mutant has growth and motility defects that are alleviated by isoleucine. The work in chapter 4 demonstrated that the branched-chain amino acid aminotransferase (IlvE) was damaged in the $_{pa}$*ridA* mutant, however, it was not determined if IlvE alone was the critical target of 2AA that led to metabolic defects. *P. aeruginosa* encodes all of the known targets of 2AA from *S. enterica* and/or *Escherichia coli*, including three glycine hydroxymethyltransferases (GlyA), alanine racemases (Alr and DadX) and a putative aspartate aminotransferase (PA3139). Nutrient analysis helped to determine the critical enzyme target of *S. enterica ridA* mutant and showed that exogenous glycine could bypass damage to GlyA (7). Aside from exogenous isoleucine, few nutrients improved growth or motility of the $_{pa}$*ridA* mutant. A slight improvement in growth was be observed from addition of glycine, vitamin B1, and proline, and polyamines the $_{pa}$*ridA* mutant. To address the 2AA enzyme targets of a $_{pa}$*ridA* mutant, a feedback resistant IlvA could be expressed *in trans* in a $_{pa}$*ridA ilvA1 ilvA2* triple mutant and growth and motility monitored in the absence and presence of exogenous isoleucine. If in a $_{pa}$*ridA ilvA1 ilvA2* exogenous isoleucine drastically improves growth or motility, these data would suggest IlvE is the critical target of 2AA damage in *P. aeruginosa.* The slight growth improvement by glycine, vitamin B1, proline, and polyamines suggest i) damage to enzymes in these pathways are not critical to growth or motility

or ii) there may be a cumulative effect of damage to several enzymes that lead to the growth and motility defects. Further analysis of the suppressor mutants could be used to probe the generation and targets of 2AA in the *P. aeruginosa ridA* mutant.

Nutritional analysis of *P. aeruginosa ridA* suppressor mutants uncovered a $_{pa}$*ridA* mutant sensitivity to valine and leucine, a phenotype that was previously overlooked. It is likely that the sensitivity of the $_{pa}$*ridA* mutant to serine, valine and leucine can be attributed to IlvA activity and generation of 2AA. As discussed in chapter 5, leucine and valine would likely activate IlvA and lead to increased 2AA generation from endogenous serine. The main generator of 2AA in *S. enterica* is serine/threonine dehydratase (IlvA), the first step in the isoleucine biosynthetic pathway. If IlvA encodes the regulatory domain, it is allosterically inhibited by binding of end product of the pathway, isoleucine, and that inhibition can be reversed by binding to valine (19-24). The first committed step in the leucine biosynthetic pathway proceeds through α-isopropylmalate synthase (LeuA, EC 4.1.3.12) and is inhibited by the end product, leucine (25). If in a $_{pa}$*ridA* mutant exogenous leucine inhibited α-isopropylmalate synthase, metabolic flux to valine would likely increase and lead to the activation of IlvA by valine. It remains to be determined experimentally if exogenous leucine or valine inhibit growth of the $_{pa}$*ridA* mutant by increasing activity to one, or both, IlvA enzymes. *In vivo* experiments with the $_{pa}$*ridA ilvA1* or *ilvA2* double mutants could determine if sensitivity to valine or leucine was dependent on one or both IlvA enzymes. *In vitro* assays could further probe each IlvA enzyme affinity for serine, valine, and/or isoleucine.

The RidA paradigm established in *S. enterica* serves as a framework to explore RidA in the metabolic contexts of diverse organisms. Study of *P. aeruginosa* and *C. jejuni* has demonstrated that RidA is integrated differently into the diverse metabolic networks and, as

expected, the phenotypic outputs vary. Despite differences in metabolic structure, i) RidA enzymes from all organisms tested have deaminase activity and ii) 2AA is assumed to be responsible for metabolic defects from damage to PLP-dependent enzymes. The conserved deaminase activity of RidA enzymes cannot be ignored, even in studies of multicellular organisms. The specific generators and targets of 2AA may differ, but phenotypic analysis of *ridA* mutants should be considered in the context of the well-establish and expanding RidA paradigm.

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