OXYGENATION IMPACTS 2-AMINOACRYLATE STRESS IN SALMONELLA ENTERICA

by

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ABSTRACT

The RidA subfamily deaminates imine/enamines, like 2-aminoacrylate (2AA). 2AA is the product of the PLP-dependent serine/ threonine dehydratase IlvA in *S. enterica*. In absence of RidA, 2AA accumulates and targets PLP-dependent enzymes, permanently inactivating them. A phenotype of 2AA stress in *ridA* mutants is the inability to grow in the presence of exogenous serine. However, *ridA* mutants grow in the presence of exogenous serine in static conditions. Phenotypes arising from 2AA stress from endogenous serine pools were not affected by aeration and lack of aeration did not allow for growth of *ridA* when other sources of 2AA were provided. Data supports that the effect of static growth is specific to increased 2AA from exogenous serine. *S. enterica* possesses an iron-sulfur cluster dependent serine deaminase SdaA. A *ridA sdaA* mutant was unable to grow in the presence of exogenous serine in static conditions. These data support a hypothesis that ROS generated during aeration are comprising SdaA, contributing to serine sensitivity.

INDEX WORDS: 2-aminoacrylate, RidA, SdaA, ROS, pyridoxal 5’ phosphate

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BS, UNIVERSITY OF MISSOURI, 2018

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2024

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DEDICATION

I dedicate this thesis to everyone who has had to have perseverance against all odds. You are worthy of respect and a fulfilled life.

ACKNOWLEDGEMENTS

I first would like to thank Diana for welcoming me into her lab and allowing me to continue my scientific journey. Thank you to me committee members for their helpful discussions. Thank you to the members of the Downs Lab, past and present, for their willingness to help me with anything and for their support. I especially would like to thank Huong and Greg for their guidance in helping me realize that I can be successful in whatever I set out to do, science related or not. Thank you to Ronnie, Brandi, and Kailey for their support throughout my time here. I would like to thank the staff of the Microbiology department for helping me with assistance regarding anything that I have needed help with.

Now, I would like to thank my best friend Reneise for encouraging me when I desperately needed it. You ae also another person that has reassured me that I can do whatever I set my mind to. Thank you for the late afternoon phone calls of encouragement. I would like to thank my older brother Terrance for being a voice of reason and a source of support for me during my time here. Last, but certain not least, I would like to thank my mother, Linda. She made the journey with me to Athens when I first started my program. We have had our fair shares of adventures and ups and downs, especially over the last 5 years, but that has never stopped her from supporting me. She has listened to me rehearse my seminar talks and helped my overall transition to being a graduate student. This journey would have been lonelier, and my life would have less sunshine without her presence. Thank for always being there for me.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The study of metabolism in varied organisms helps us as scientists to expand our understanding of how they can survive in their respective environments. This knowledge has especially helped with the field of bacteriology. The ability to map the genomes of these bacterial organisms has not only led to a better understanding of these microorganisms, but scientists have been able to notice similarities and differences between different domains of life. One goal of research in the Downs' lab is to identify the connections that exist between metabolic pathways within the model organism, *Salmonella enterica*. By defining these connections, we can extend these findings to better understand how these metabolic pathways work in similar and distinct organisms.

Our studies of the Rid Superfamily/RidA Subfamily, a group of small proteins that are conserved in all domains of life, has provided a chance to further these discoveries (1). Our findings show not only how RidA functions within the metabolism of *S. enterica*, but also how RidA functions within other closely related organisms. Close to 30 years of findings with biochemical, genetic and technological advancements has led the current understanding of the importance of RidA is to the metabolic network of multiple organisms (1-4). It is fascinating to see how a small protein is relevant to the overall functioning of organisms that have survived for millions of years. As the efforts of our lab and others continues to increase our understanding about RidA and other Rid proteins in other organisms, we are still expanding our knowledge about these proteins in original model organism, *S. enterica*. The RidA Paradigm, while an important piece in

understanding it's function, does not begin to tell the whole picture of how the loss of RidA impacts the metabolism of *S. enterica*.

The following literature review delves more into the background of the Rid Superfamily, with the emphasis on the RidA Subfamily. The following sections will also explore into reactive intermediates such as 2-aminoacrylate, as well as how the RidA Paradigm was elucidated, and the methods used to identify the impacts of the loss of RidA within the metabolic pathways of S. enterica. This background is the basis of the thesis project performed, which will be described in Chapter 2.

1.1. THE RID SUPERFAMILY/RIDA SUBFAMILY

The discovery of small proteins over the last century has unlocked more questions than answers about the importance of these proteins throughout evolution. The rise of technological advances in sequencing and genetic methods has allowed for the identification of the Rid Superfamily of proteins and why these proteins have survived through years of evolution. The Rid (YjgF/YER057c/UK11) superfamily consists of proteins of previously unknown function (1-4). Sequence analyses from bacteria, archaea, and eukaryotes showed homology with some of the proteins from this large family. In brief, after comparing these sequences, the superfamily was grouped into smaller subfamilies. The RidA (which is the focus of this thesis), Rid1-3, Rid4-7, and RutC subfamilies have distinct sequence features (1, 5). The original interest behind this superfamily was their previously uncharacterized function(s). The fact that these proteins are conserved in all domains of life, while not having a known function, showed that they must have an important role the metabolism of these organisms.

Before studies that would lead to the elucidation of the RidA Paradigm of 2AA Stress in *S. enterica,* members of the RidA subfamily had been assigned potential functions that were highlighted in studies in a variety of organisms. In 1993, RidA was isolated from rat liver. RidA was found to be soluble in perchloric acid and showed homology to heat shock and/or chaperone proteins (6-12). While these functions that were predicted were important, there was insufficient evidence to conclude they wer the only, and or relevant, functions. Based on this study (mentioned earlier in this section), homologs of RidA were found in several organisms and were tested. Some of the homologs were *Salmonella enterica* RidA, *Escherichia coli* RidA and TdcF, *Pseudomonas aeruginosa* RidA, *Cucumis sativus* ChrD, and *Capra hircus* UK114 (1, 13-17).

In comparison to the other Rid proteins, RidA (based on crystal structures in *E. coli*), has an important Arg105 residue that can form bonds with a substrate enamine and imines (described in depth in Sections 1.3) (17-18). While this residue is universally conserved in RidAs, it was also found to be present in the Rid1-3 and RutC protein subfamilies as well and is essential for deaminase activity (18-20). Rid4-7 proteins, to date, are the only subfamily that do not possess the important Arg105 residue (18, 21). As of now, Rid4-7 are not known to be able to use enamine and imines as substrates based on the lack of the Arg105 (1, 22). In all, the Rid Superfamily consists of proteins with similar and different properties. To date, RidA is the most characterized of the superfamily, and more information is being uncovered through current studies.

1.2. REACTIVE METABOLIC SPECIES AND INTERMEDIATES

Biochemical pathways generate intermediates metabolites on the way to synthesizing a desired end-product. Some intermediates are known to be toxic or reactive, and others are not. Well-known intermediates and end-products are reactive oxygen species (ROS) and reactive nitrogen species (RNS) (3, 23). ROS are known to be produced in the process of reduction of molecular oxygen by the abstraction of electrons, which can lead to oxidative stress (24-25). Briefly, molecular oxygen (O_2) is reduced first to superoxide (O_2-), then superoxide is reduced to hydrogen peroxide (H_2O_2), while hydrogen peroxide is reduced to hydroxide (OH-), and then hydroxide is reduced further to water (H_2O) (25-27). The two most studied ROS that are produced in this process are superoxide and hydrogen peroxide. Superoxide is known to damage certain iron-sulfur cluster (2Fe-2S or 4Fe-4S) dependent enzymes, which prevent them from carrying out their metabolic duties and can affect the way sulfur can be used as the sole sulfur source (25-28). Hydrogen peroxide, while not as reactive as superoxide, can cause damage to DNA even in a brief exposure and there is some data to suggest that hydrogen peroxide could have a negative effect on iron-sulfur cluster dependent enzymes (25, 29-33). RNS can be made by an organism in hopes of deterring invading hosts. However, these species can also still be toxic within the organism. Some well-studied RNS are nitric oxide (NO), nitrite (N_2O_3), and peroxynitrate (ONOO-) (34-38). Nitric oxide is often the most abundant RNS in most organisms and it used to generate other RNS. The enzymes that are known to produce nitric oxide are NO synthases (NOS) (35-37).

Other reactive or non-reactive species can be produced within certain metabolic biosynthetic pathways. Of particular interest herein are intermediates that are produced by the threonine/serine degradations dehydratases (4, 39-40). Using these amino acids as substrates produces an enamine/imine tautomerization that includes the enamines 2-aminocrotonate (2AC) or 2-aminoacrylate (2AA), from threonine and serine respectively. These reactive intermediates are converted to either to 2-ketobuyrate (2KB) and pyruvate, respectively (4). Enamines and imines are metabolites that have a short half-life and multiple enzymes can catalyze the formation of these metabolites. The basis of this section is to highlight certain reactive intermediates and species that will be important not only to this thesis, but the basis of all work that have been done on the RidA Paradigm of 2AA stress over the past 15 years.

1.3.THE RIDA PARADIGM OF 2AA STRESS

Elucidation of the RidA Paradigm in *S. enterica* started from a study that was unrelated to RidA. Using genetic and biochemical approaches in thiamine biosynthesis, RidA was found to play a significant role in this biosynthetic pathway. Briefly, a *purF gnd* mutant is unable to synthesize the pyrimidine moiety of thiamine (41-45). Importantly, the *gnd* mutation does not allow for growth on minimal glucose with adenine and pantothenate, while the single *purF* mutation can grow in this medium (41). The addition of the *gnd* mutation suggested that the pentose phosphate pathway might have had a role in thiamine biosynthesis. Suppressor mutations of *purF gnd* were isolated on minimal glucose plates with adenine and pantothenate. After careful identification of mutants isolated, a mutation mapped to *yjgF* (to be renamed *ridA*) was identified (41-45).

The null mutation of *ridA* was found to restore the ability of *purF gnd* to grow on minimal glucose with adenine and pantothenate. The *ridA* was cloned into a plasmid, where it was confirmed to complement the phenotype of *purF gnd* on minimal glucose with adenine and pantothenate (41-45). Further database analysis found RidA to have several homologs across bacteria and other domains of life (41).

While this study was informative for thiamine biosynthesis, another phenotype of a *ridA* phenotype was discovered. A single *ridA* mutant was found to have no growth on minimal glucose medium with serine supplementation. Testing metabolites that could potentially rescue this phenotype, addition of isoleucine to minimal serine medium was found to rescue growth (41). This suggested that isoleucine biosynthetic pathway was important to suppression of the *gnd* mutation in thiamine synthesis, however, a study in 2004 proved that the isoleucine requirement for a *ridA* mutant to grow with serine was separate from thiamine biosynthesis (46).

The next study dove deeper into the phenotype that a *ridA* mutant could only grow in minimal serine medium when isoleucine was present. From the previous data, it was suggested that the isoleucine biosynthetic pathway was impacted in the absence of *ridA*. To test this, a biochemical assay which tested the activity of Transaminase B (IlvE EC;2.6.1.42) was used (46). IlvE is the last step in isoleucine biosynthesis that converts 2-keto-3-methylvalerate (2KMV) to isoleucine via the transamination process of L-glutamate and 2-ketoglutarate (2KG) (46-48). Shockingly, in the *ridA* mutant there was approximately half the IlvE activity of the wildtype strain when measured in crude extracts (46). When isoleucine was added to growth medium and IlvE activity was assayed again, IlvE activity in *ridA* mutants was restored to that of the wildtype strain. To

understand if a *ridA* mutant was sensitive to metabolites in the isoleucine biosynthetic pathway, two experiments were conducted. Growth data to test if *ridA* was sensitive to metabolites in the pathway such as 2-ketobuyrate (2KB), the product when threonine was used as a substrate by the threonine deaminase IlvA (threonine/serine dehydratase EC;4.3.1.19), showed that *ridA* was not sensitive to this metabolite (46). However, data showed that IlvA was responsible for IlvE activity being lowered in *ridA* and addition of isoleucine allosterically inhibited IlvA. This was confirmed using a feedback resistant variant of IlvA (explained in Section 1.4) (46). At this point, a hypothesis was formed that suggested that RidA might be needed in the threonine degradation/dehydratase pathway, in accordance with the above data.

IlvA is known to produce enamine/imine intermediates while using threonine or serine as a substrate, ultimately producing the ketoacids 2-ketobuyrate and pyruvate, respectively (39, 47, 49). There was merit in thinking that RidA could have a role in quickly producing these ketoacids, potentially to rid the cell of these enamine/imine intermediates (19, 49). A study was conducted to test this hypothesis. Biochemical assays showed that not only was RidA was able to increase the formation for 2KB and pyruvate, but that RidA decreased the availability of enamine/imine intermediates (19). The fact that a *ridA* mutant was sensitive to serine and RidA protein was able to decrease the amount of the enamine/ imine intermediate associated with IlvA using serine as a substrate (2-aminoacrylate), suggested that RidA acted on enamine/imine intermediates to increase the rate of formation of ketoacids (19). When RidA is absent, these ketoacids are made slower and 2AA accumulate in the cell.

1.4. DAMAGE OF PYRIDOXAL 5' PHOSPHATE ENZYMES

Previous studies showed that the PLP-dependent enzyme IlvA generates 2AA by using serine as the substrate (1, 19). 2AA is then deaminated by RidA to generate the ketoacid pyruvate. The hallmark phenotype of a *ridA* mutant is the inability to grow in minimal glucose medium with exogenous serine supplementation (41). Addition of exogenous isoleucine allows for a *ridA* mutant to grow in the medium (41). This discovery led to the assumption that isoleucine biosynthesis could have been disrupted. At this point, two enzymatic steps in isoleucine biosynthesis were assayed to know if this was the correct assumption. Threonine deaminase (IlvA) and Transaminase B (IlvE) activities were assayed in wildtype and *ridA* strains. Threonine deaminase activity between the two strains were not significantly different, however, the IlvE activity of the *ridA* mutant was significantly lower than the wildtype (41). The addition of isoleucine to the growth medium increased IlvE activity in *ridA* strains. At first, the results could have been interpreted by the absence of *ridA* causes a nutritional requirement for isoleucine, however, additional studies would provide more information on how RidA deaminates the reactive intermediate 2AA and isoleucine addition allosterically inhibited IlvA from generating 2AA. This study was the catalyst of understanding that certain PLP-dependent enzymes are damaged in a *ridA* mutant.

One of the next studies provided the context that RidA increases the rate of 2-ketobuyate (2KB) and pyruvate formation (19). This important observation led to the experiment that involved testing the ability of RidA to deaminate enamine/imine intermediates. RidA could in fact deaminate these intermediates, which was then

proposed as the function of RidA. RidA is needed to deaminate these reactive intermediates and that this is the conserved function of the RidAs tested from in all domains of life. From further studies, 2AA was found to permanently inhibit certain PLP-dependent enzymes like IlvE unless RidA was present to promptly deaminate the reactive species to a ketoacid (2). While this was an incredible observation, which added to the paradigm, more needed to be understood about the capacity of 2AA to attack PLP-dependent enzymes and if those are the only cofactor enzymes that were attacked by 2AA.

A study that was initiated by the observation that *ridA* could not grow on L-alanine as a nitrogen source. That study concluded that the reasoning behind the phenotype was the fact that alanine racemases Alr and DadX (EC; 5.1.1.10) were damaged by 2AA, which inhibited the conversion of L-alanine to D-alanine, which is then converted by DadA to pyruvate (50-53). At this point, there was evidence that a pyruvate/PLP adduct was formed on Alr (1, 52). For the first time, there was evidence that 2AA was the cause of damage to more than one PLP-dependent *in vivo* and that this damage renders the enzymes inactive (1, 52). Based on the *in vitro* findings, there was a possibility that other PLP-dependent enzymes could be inactivated in the cell, which propelled studies further.

One of the studies showed that there was a decrease in the activity of serine hydroxymethyltransfrase GlyA (EC; 2.1.2.1). GlyA is an essential enzyme that catalyzes serine into glycine by generating methylene-tetrahydrofolate from tetrahydrofolate (THF), which can be used further downstream in various pathways such as one-carbon metabolism or pantothenate biosynthesis (52). This discovery was aided by a previous

phenotype that showed a *ridA* mutant's inability to grow on minimal pyruvate medium. Briefly, a *ridA* mutant was found to accumulate pyruvate in growth medium, which suggested that there was a decrease in coenzyme A (CoA) levels (54). As the study concluded, the reason that CoA levels were decreased was because of a decrease in GlyA activity in *ridA*, causing a defect in one-carbon metabolism (54). GlyA activity was more than 80% decreased in a *ridA* mutant (55). Expanding on the findings in this study, GlyA was more intensely studied. More experiments saw that glycine addition to minimal serine medium also helped a *ridA* mutant grow (55-56). An interesting *in vitro* result suggested a new perspective on the importance of GlyA. Glycine addition, unlike isoleucine addition, did not improve the IlvE Activity of a *ridA* mutant (52, 55-56). Even though glycine prevented the growth defect, it did not result in lessened the 2AA stress in a *ridA* mutant (41,55-56). In combination, results from the two previous studies determined that GlyA is the most important enzyme in a *ridA* mutant that is damaged in *S. enterica*, given the different defects that are caused by the loss of RidA that are directly connected to GlyA damage (52, 55-56).

In conclusion, studies showed that certain PLP-dependent enzymes are attacked and inactivated by 2AA. A pyruvate/PLP adduct is made from 2AA attacking PLP. To date, there have been only a handful of PLP-dependent enzymes shown to be inactivated by 2AA, with a possibility of more to be named. The first PLP-dependent enzyme that was found to be attacked by 2AA was IlvE. Studies that followed identified more enzymes such as alanine racemase Alr/DadX and GlyA. GlyA is thought to be the most important PLP-dependent enzyme that is inactivated by 2AA because of the multiple defects caused by inactivated GlyA in *ridA* mutants. Briefly mentioned here, but another

enzyme that is inactivated by 2AA is AspC (1, 57-62). More PLP-dependent enzymes are thought to be inactivated, and future studies should continue to investigate. Most evidence points towards certain PLP-dependent enzymes (based on fold type) are damaged by 2AA (1,63-66).

1.5. EXPANSION OF RIDA PARADIGM PHENOTYPES

The first phenotype associated with 2AA stress in *S. enterica* was the inability to grow in minimal glucose medium with exogenous serine. Throughout the studies of the RidA Paradigm, more phenotypes have arisen that have informed more about the impact that 2AA stress has on the greater metabolic network. In addition to inhibited growth of *ridA* on minimal serine medium, a *ridA* mutant was also found to not be able to grow on minimal medium with exogenous cysteine or 2'3- diaminopropionate medium (55-56, 67). These observations helped to elucidate that there were other generators of 2AA stress in *S. enterica.* These generators are CdsH (cysteine desulfhydrase, EC; 4.4.1.1) and DapL (2'3 diaminopropionate ammonia-lyase, EC; 4.3.1.15), along with IlvA (55-56, 67). Simple growth experiments that provide these phenotypes have been the catalyst for discovering much about the functions of RidA in the cell.

Mentioned in an earlier section, the addition of exogenous isoleucine to minimal serine medium allows for growth of a *ridA* mutant due to allosteric inhibition of IlvA. As other phenotypes of a *ridA* mutant were being explored, a *ridA* mutant was found to not be able to grow on minimal pyruvate medium (68). Like growth on minimal serine medium, a *ridA* mutant was only able to grow on minimal pyruvate medium when exogenous isoleucine was added (68). This observation indicated that the generation of

2AA was stopped and for a reason unknown at the time, *ridA* was needed for growth when using pyruvate as a carbon source. A feedback resistant variant on IlvA (L447F) was utilized to see if IlvA, thus 2AA generation, was the cause of this phenotype (46, 68-69). The variant has a mutation in the regulatory domain that prevents isoleucine from allosterically inhibiting IlvA. The data from this study concluded that exogenous isoleucine rescued growth of a *ridA ilvA219* strain, meaning that the loss of *ridA* impacted isoleucine biosynthesis separate from 2AA stress (68-69). The isoleucine requirement was a consequence of IlvE activity being decreased because of 2AA attacking and inhibiting the PLP-dependent enzyme (46, 68).

The observation that a *ridA* mutant could not use L-alanine as a nitrogen source led to the study that showed PLP-dependent alanine racemases are also damaged by 2AA. In this study, a *ridA* mutant could only grow on minimal NCN medium with L-alanine as sole nitrogen source only if exogenous isoleucine was added to the medium (54, 70). Again, the fact that this phenotype was corrected by isoleucine implicates 2AA stress impacting a part of the metabolic network that was not previously seen before. The literature suggested that the inability of a strain to grow using L-alanine as a nitrogen source could be caused by damage to alanine racemase DadX (54, 70). DadX and Alr, like IlvE, are PLP-dependent enzymes, so it was likely that they were damaged by 2AA. The conclusions from one study showed that Alr was more damaged in a *ridA* background compared to a wildtype background. In addition, when Alr was purified from both *ridA*$^{+}$ and *ridA*$^{-}$ background, Alr from the *ridA*$^{-}$ background contained a pyruvate/PLP adduct, which was the first evidence of a 2AA/PLP adduct being generated *in vivo* (54, 70).

From our later studies, more phenotypes of 2AA stress in a *ridA* mutant have been uncovered such as a motility defects (1,57). As more information is discovered about the wider importance of *ridA*, using phenotypic experiments will and has become crucial. Without these simple, yet important experiments, the first observation of a *ridA* mutant would not have been noticed. As this thesis continues in subsequent chapters, the importance phenotypes that were observed provided context for the model that was proposed.

1.6. SERINE DEGRADATION

The discovery that a *ridA* mutant cannot grow on minimal glucose medium with serine supplementation, while consistent with RidA Paradigm, leaves more questions than answers. Why is serine not being degraded in a timely matter to prevent this phenotype? The answer could rely on the serine degradation process outside of the confines of how RidA interacts with the entire metabolic system of *S. enterica*. Like *E. coli*, *S. enterica* has multiple serine deaminase/dehydratases. As mentioned in previous sections, IlvA is one of the enzymes that can use serine as a substrate, which generates 2AA. However, there are several enzymes that can deaminate or dehydrate serine with or without the release of 2AA (49, 71).

TdcB (catabolic threonine dehydratase, EC 4.3.1.19) is one enzyme that degrades serine into pyruvate by generating 2AA (72-77). Like IlvA, TdcB is a PLP-dependent enzyme, but has some differing properties compared to IlvA (72-77). There have been multiple reports that indicate *tdcB* is only expressed in anaerobic conditions (78-85). However, there have been reports and data that show this might not be the case. In any

case, TdcB is a major enzyme that is known to degrade serine. Other enzymes that degrade serine are enzymes with an active 4Fe-4s clusters. These iron-sulfur dependent enzymes can be attacked by ROS, as mentioned in a previous section. Given the sensitivity of Fe-S clusters ROS, these enzymes are expected to be the most active in anaerobic conditions. Like IlvA and TdcB, these enzymes produce the ketoacid pyruvate (68, 86-88).

In *S. enterica*, Fe-S cluster serine dehydratase enzymes are SdaA (serine deaminase I, EC 4.3.1.17), SdaB (serine deaminase II, EC 4.3.1.17), and TdcG (serine deaminase III, EC 4.3.1.17). TdcB and TdcG, although are part of the same operon of genes and are thought to both be anaerobically activated, have different cofactors (PLP and 4Fe-4S) (85, 88-91). SdaA and SdaB are found in both *S. enterica* and *E. coli*. SdaC (L-serine: H+ symporter), which is in an operon with SdaB, is thought to be a serine H+ symporter in both *S. enterica* and *E. coli*. Interesting, *sdaA* is negatively regulated by Lrp and Nac and is not located in the operon with *sdaB* and *sdaC*. Lrp (leucine-responsive regulatory protein) is a well-studied DNA-binding transcriptional dual regulator that is known to regulate 10% of genes in *E. coli* as a repressor or activator (92-95). Leucine inhibits Lrp, which prevents Lrp from negatively or positively activating genes. Nac (nitrogen assimilation control) regulates genes in nitrogen metabolism when there is a nitrogen limiting condition (96-99). Nevertheless, SdaA and the rest of the enzymes that are known to degrade serine are important for maintaining the metabolic environment of organisms (100).

1.7. OUTLINE OF THESIS

The basis of this thesis was to expand upon the knowledge on 2AA stress. In particular, I sought to probe differences in the way that a *ridA* mutant responses to 2AA stress in different growth conditions. Previous studies have shown that a *ridA* mutant is sensitive to exogenous addition of serine in growth medium. This is due to the abundance of 2AA accumulation in the cell. However, while this is true for certain growth conditions (normal laboratory shaking conditions), this is not necessarily true for all growth conditions.

In the following chapter, data will show that a *ridA* mutant can grow successfully in minimal serine medium in static (non-aerated) conditions. This discovery is quite the departure from what has been known about a *ridA* mutants. However, despite this observation, the data also show that 2AA stress still does have an impact on a *ridA* mutant in non-aerated environments. Data shows that seemingly only 2AA stress is suppressed only when *ridA* is grown in minimal serine medium, not other generators of 2AA stress (exogenous cysteine and 2’3-diaminoproprionate). These observations led to the hypothesis that there were issues in serine degradation in shaking (more aerated) environments. A model developed in which iron-sulfur [4Fe-4S] cluster dependent enzymes that are responsible for degrading serine, such as SdaA, could be damaged by ROS. This could prevent exogenously added serine that is taken up in the cell from being properly degraded, leading to accumulation of 2AA in a *ridA* mutant. Further experiments in static conditions have provided data that supports this model. Overall, this thesis not only expands the scope of how RidA is important to the metabolic network of

S. enterica but has also expanded how interconnected the metabolic network and disrupting multiple parts of the network causes a myriad of issues that are sometimes hard to see.

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CHAPTER 2

OXYGENATION IMPACTS 2-AMINOACRYLATE STRESS IN SALMONELLA ENTERICA[1]

1

[1] Collier, A.L. and Downs, D.M. To be submitted to a peer-reviewed journal

ABSTRACT: Members of the RidA subfamily deaminate imine/enamines, like the reactive species 2-aminoacrylate (2AA). 2AA is the product of the PLP-dependent serine/ threonine dehydratase IlvA in *Salmonella enterica*. In the absence of RidA, 2AA accumulates and targets certain PLP-dependent enzymes, rendering them permanently inactivated. A diagnostic phenotype of 2AA stress in a *ridA* mutant is the inability to grow in the presence of exogenous serine. During other studies, it was noted that *ridA* mutants grew in the presence of exogenous serine when incubated in static conditions. This study was initiated to understand this phenomenon. Phenotypes that arise from 2AA stress derived from endogenous serine pools were not affected by the level of aeration and lack of aeration did not allow for growth of a *ridA* mutant when other sources of 2AA were provided. The data supported the conclusion that the effect of static growth was specific to increased 2AA resulting from exogenous serine. *S. enterica* possesses an iron-sulfur cluster dependent serine deaminase SdaA. The sensitivity of iron-sulfur clusters to reactive oxygen species (ROS) suggested a role for SdaA in the relevant phenotypes. A *ridA sdaA* mutant was unable to grow in the presence of exogenous serine in static conditions but importantly, growth was restored by addition of isoleucine, connecting the serine sensitivity to generation of 2AA by IlvA. These data support a hypothesis that ROS generated during growth with aeration are comprising SdaA, resulting in an increased internal concentration of serine and increased 2AA stress in a *ridA* mutant. These results increased our understanding of the conditions that result in 2AA stress in *S. enterica* and identified a new player in the metabolic network impacting this stress.

INTRODUCTION: The Rid (YjgF/YER057c/UK114) protein superfamily is divided into nine subfamilies (1). The RidA (reactive intermediate deaminase A) subfamily are found in all domains of life, whereas Rid1-7 and RutC subfamilys are found exclusively in various prokaryotes. RidA homologs have appeared in the literature for over 30 years (2). The RidA paradigm of 2-aminoacrylate (2AA) stress was constructed based upon studies in *Salmonella enterica* (2-6). Studies found that RidA deaminates 2AA generated from three enzymes: serine/threonine dehydratase (IlvA, EC 4.3.1.19), cysteine desulfhydrase (CdsH, C. 4.4.1.15), and diaminopropionate ammonia-lyase (DapL, EC 4.3.1.15) (7-10). These enzymes use serine, cysteine and 2,3-diaminopropionate (Dap), respectively, as substrates to generate the 2AA intermediate (8-10). In the absence of RidA, 2AA accumulates and attacks certain pyridoxal 5'-phosphate (PLP)-dependent enzymes such as branched-chain amino acid aminotransferase (IlvE, EC 2.6.1.42), serine hydroxymethyltransferase (GlyA, EC 2.1.2.1), and alanine racemase (Alr/DadX, EC 5.1.1.1) (2, 7-11). The enzymes are inactivated by the generation of a 2AA/PLP adduct covalently bound to the enzyme. RidA proteins with similar activity have been described in other bacteria including *Pseudomonas aeruginosa* (PA5339), *Escherichia coli* (RidA and TdcF), and *Campylobacter jejuni* (Cj1388), to name a few (2).

Previous studies have noted that a *ridA* mutant in *S. enterica* do not grow in the presence of exogenous serine, cysteine, and Dap in minimal glucose medium. This phenotype was demonstrated to be due to the accumulation of 2AA in the cell. Exogenous isoleucine added to minimal medium with exogenous serine rescues the growth, due to allosteric inhibition of IlvA, thus alleviating 2AA stress. The resulting decrease in 2AA has also been shown by assaying IlvE activity as a proxy for 2AA damage and stress. A

ridA mutant has ~50% less IlvE Activity due to damage of IlvE by 2AA (7). Addition of isoleucine increases the activity of IlvE to near wildtype levels (7). Exogenous glycine addition to minimal medium with exogenous serine, cysteine, and Dap rescues growth (2, 7-11). Suppression by glycine is due to its ability to circumvent the damage to GlyA by 2AA, which is the primary target of 2AA in a *S. enterica ridA* mutant (10-11).

It was previously observed that a *ridA* mutant could grow in the presence of exogenous serine in the absence of aeration, which is different from the standard shaking conditions used in the laboratory. The main difference between the two conditions is the presence of oxygen, leading to possibility that oxygen impacted the synthesis or consequences of 2AA in *S. enterica*. This study was initiated to understand this phenomenon. The data herein support a model in which aeration compromises the removal of serine, possibly through ROS targeting the Fe-S clusters in SdaA. Different growth conditions with and without oxygen provided the hypothesis that a *ridA* mutant could be more susceptible to reactive oxygen species (ROS), which can attack Fe-S cluster dependent enzymes like the serine deaminase SdaA (EC 4.3.1.17) (12). Our hypothesis is that damage to SdaA by ROS, prevents the enzyme from detoxifying exogenous and endogenous serine in shaking conditions in a *ridA* mutant. In static conditions with less oxygen, SdaA is less susceptible to ROS and can detoxify serine, allowing a *ridA* mutant to grow. This study provides insights into the importance of RidA in impacting the greater metabolic pathways in *S. enterica*.

RESULTS:

Static growth alleviates serine sensitivity in *ridA* mutants. In the laboratory, *S. enterica* is standardly cultured in minimal glucose medium with shaking to increase aeration. In these conditions, a *ridA* mutant is unable to grow when 5 mM serine is in the medium. However, when incubated in the same medium without shaking, a *ridA* mutant grew to the same density as the wild-type strain (OD_{650} ~0.5, Table 1). Differences between the two conditions that could explain the difference in growth were considered. *S. enterica* use glucose as a carbon and energy source by respiration or fermentation. In the absence of aeration, available oxygen is concentrated at the liquid/air interface and is rapidly consumed, leaving the majority culture growth under oxygen depleted conditions. Without an alternative electron acceptor, this condition would lead to fermentation, suggesting serine sensitivity of a *ridA* mutant could depend on the mode of energy generation. Wildtype and *ridA* strains were grown with and without aeration when glycerol was the carbon source and nitrate provided as an alternative electron acceptor. The *ridA* mutant was sensitive to serine in minimal glycerol medium when cultures were aerated (OD650 ~0.1, Table 2), but not when grown statically (OD_{650} ~0.3, Table 2). These data ruled out a scenario in which serine sensitivity occurred only during respiration.

In a simple scenario the relevant difference between growth with and without aeration is the level of oxygen. A significant difference in oxygen levels between the two conditions was confirmed by assessing the growth of a *metE* mutant of *S. enterica*. Strains lacking MetE (EC. 2.1.1.14) fail to grow in the presence of oxygen unless supplemented with vitamin B12 or methionine. Lack of oxygen allows *S. enterica* to synthesize B12 and thus use MetH (EC. 2.1.1.13) to generate methionine (13-14) The *metE* mutant required methionine to grow in aerated cultures, OD_{650} ~0.1 without Met vs. OD_{650} ~1.2 with Met

(Table 1). The same strain grew to full density without the addition of methionine in static conditions (OD_{650} ~0.6, Table 1). These data confirmed cultures grown in the absence of shaking were limited for oxygen.

Serine is distinct from other 2AA generators. A *ridA* mutant is sensitive to exogenous cysteine and 2,3-diaminopropionate (Dap), which generate 2AA from CdsH and DapL (EC 4.4.1.1/ EC 4.3.1.15) respectively (9-10). If lack of aeration impacted the effect of 2AA, a *ridA* mutant would not be expected to be sensitive to cysteine and Dap under this condition. Contrary to this expectation, a *ridA* mutant was equally sensitive to both exogenous cysteine and Dap when grown with and without aeration (OD_{650} ~0.1 or 0.2, respectively) (Figure 2). These data suggested that a property of 2AA stress specific to serine and/or IlvA was involved in the aeration-specific phenotypes of the *ridA* mutant.

2AA stress is present in statically grown cultures. In general, mutations and media that suppress consequences of a *ridA* mutant either reduce the amount of 2AA (i.e. isoleucine) or bypass a key enzyme damaged by 2AA (i.e. glycine). These two possibilities can be separated by the level of 2AA in cell. Since 2AA cannot be measured directly due its rapid turnover. The activity of branched chain aminotransferase (IlvE) is an established proxy for the level of 2AA stress in the cell. IlvE activity was assayed in crude extracts of cells grown under static vs shaking conditions (Table 3). When grown in minimal medium with shaking, a *ridA* mutant had ~80% the IlvE activity of the wildtype strain (WT, 472± 11 and *ridA*, 314± 8 nmol/mg, Table 3), consistent with what has been noted in previous studies (7). When grown in minimal medium in static conditions, a *ridA* mutant similarly had a reduced level of IlvE activity (WT, 376± 1 and *ridA*, 207± 1, Table 3). These data suggest that 2AA stress in present even in static growth conditions.

Endogenous 2AA defects are not alleviated by static growth conditions. Our next question was if the effects of endogenous serine and 2AA are alleviated in *ridA* mutants grown in static conditions? It was easy to assume that endogenous 2AA effects could still influence a *ridA* mutant grown statically, based upon the IlvE activity data in static conditions (Tables 3 and 4). Our previous studies show that endogenous 2AA affects the ability of a *ridA* mutant to use pyruvate as a carbon source (7, 15-16). This is due to damage of IlvE that disrupts isoleucine biosynthesis (7, 15-19). Addition of exogenous isoleucine rescues the phenotype in pyruvate, an observation separated from allosteric inhibition of IlvA (16). To test if the strain could use pyruvate as a carbon source, a *ridA* mutant was grown in minimal pyruvate medium with nitrate as an electron acceptor in shaking and static conditions, with and without addition of exogenous isoleucine. A *ridA* mutant could not grow with pyruvate as a carbon source in static conditions (OD_{650} ~ 0.1-0.2, Figure 4) and growth was allowed by exogenous isoleucine (OD_{650} ~ 0.5, Figure 4). These data suggest 2AA generated from endogenous serine pools still has effects on a *ridA* mutant in static conditions, which was consistent with the IlvE activity assay data.

Another instance where endogenous serine affects a *ridA* mutant is by causing a lack of growth on minimal NCN glucose media with L-alanine used as a nitrogen source (11). As we have noted previously, an *alr ridA* mutant cannot grow using L-alanine as a nitrogen source due to damage of alanine racemase DadX (EC 5.1.1.1) (11). Only when exogenous isoleucine is added to growth media can a *ridA* mutant grow under these conditions. We wanted to know if *an alr ridA* mutant could grow using L-alanine as a nitrogen source in static conditions? It was expected that an *alr ridA* mutant could not grow using L-alanine as a nitrogen source due to the data shown with a *ridA* mutant not being

able to use pyruvate as a carbon source static. However, there was poor growth in general with not only the *ridA* strain, but wildtype as well using L-alanine as a nitrogen source statically, so this could not be properly explored (data not shown).

Lack of SdaA exacerbates serine sensitivity in *ridA* mutants. There was merit in thinking that under aerated conditions ROS could attack a prominent 4Fe-4S dependent enzyme that could use serine as a substrate. One enzyme that was explored was the serine deaminase SdaA (EC 4.3.1.17). SdaA is one of three known 4Fe-4S dependent serine deaminases in *S. enterica* that uses serine as a substrate to produce pyruvate, without releasing 2AA (10). SdaA is also the primary source of this activity in the presence of glucose, while the other enzymes are carbon catabolite repressed (20-35). We explored the hypothesis that ROS-mediated damage to SdaA contributed to the serine sensitivity of a *ridA* mutant in shaking conditions. To do this, we sought to mimic aeration by deleting *sdaA*. A *ridA sdaA* mutant was generated and grown in static conditions, with and without addition of serine. By removing SdaA in *ridA* mutant and growing the double mutant in static conditions, this could mimic a *ridA* mutant grown in shaking conditions if our hypothesis that SdaA is damaged is correct. If our hypothesis was correct, then a *ridA sdaA* mutant would not be able to grow well in the presence of exogenous serine statically. Our experiment showed that in fact a *ridA sdaA* mutant could not grow well in the presence of exogenous serine (OD_{650}~ 0.2, Table 6). The only time the mutant could grow to full density in the presence of serine was after the addition of exogenous isoleucine, implicating 2AA stress alleviation (OD_{650}~ 0.8, Table 6). The data show that possible damage to SdaA prevents the enzyme from detoxifying exogenous serine in shaking conditions in a *ridA* mutant.

DISCUSSION:

The basis of this study was to examine the differences of serine sensitivity of a *ridA* mutant based on environments with and without oxygen present. A *ridA* mutant is known to not be unable to grow in minimal media with exogenous serine when aerated. In shaking conditions, there is more oxygen present. However, in static conditions where there is less oxygen present, a *ridA* mutant grows to full density in the presence of serine. Our previous studies have shown that the reason why a *ridA* mutant is sensitive to serine is because of serine being used as a substrate for the PLP-dependent threonine/serine dehydratase IlvA to generate the reactive imine/enamine intermediate 2-aminoacrylate (2AA). 2AA is known to damage and permanently inactivate certain PLP-dependent enzymes such as GlyA, IlvE, and AspC (1-19). The RidA Paradigm of 2AA Stress was elucidated, partly based upon these studies. 2AA can be generated using three different enzymes to date: IlvA, CdsH, and DapL. CdsH uses cysteine as a substrate and DapL uses Dap has a substrate to generate 2AA (9-10). 2AA is supposed to be deaminated by RidA. The deletion of *ridA* makes 2AA accumulate and similarly like the addition of exogenous serine, adding exogenous cysteine and Dap prevents growth of a *ridA* mutant (9-10). To determine if a *ridA* mutant's ability to grow in minimal serine medium was tied to lessening of 2AA stress, *ridA* was grown in minimal glucose medium with the addition of exogenous cysteine and Dap in static conditions. A *ridA* mutant was not able to grow in the media with the additions. From this experiment, it was determined that a *ridA* mutant was able to grow with only one generator of 2AA, not the other known

generators. At this point, it was possible that this phenotype was not based upon 2AA stress at all.

Before that determination could be made however, there were other ways to explore if the effect of 2AA stress was lessened in static conditions. In previous studies, we noted that there two simple ways to determine if 2AA stress is present in *ridA* mutants. One way is to grow a *ridA* mutant in minimal pyruvate medium. Briefly mentioned before, a *ridA* mutant cannot grow with pyruvate as a sole carbon source due to damage of the branched-chain amino acid transferase IlvE (7). IlvE is damaged by 2AA, which causes a nutritional defect in isoleucine biosynthesis, due to the last step in the pathway being inhibited (transaminase reaction to produce isoleucine by IlvE). The lack of isoleucine production causes accumulation of pyruvate within the cell. A *ridA* mutant cannot grow with pyruvate as a carbon source because of this. A *ridA* mutant was grown in minimal pyruvate media under static conditions to see if the impact of 2AA stress was lessened. If this were the case, *ridA* would have been expected to grow. However, *ridA* was not able to grow on minimal pyruvate in static conditions unless exogenous isoleucine was added. Another way to show if 2AA stress was present was assaying IlvE Activity. Like in shaking conditions, a *ridA* mutant had less IlvE activity in static conditions. Even though *ridA* can grow with serine in static conditions, these results showed that 2AA stress is not lessened in static conditions.

The results that showed that 2AA stress was still present in *ridA* mutants grown statically made the initial results slightly perplexing. Why was 2AA stress not eliminated? The simple fact that serine sensitivity was not determined by ability to respire suggested that oxygen was the key component in determining growth in the

presence of serine. Knowing that only serine addition is impacted between environments planted the idea that serine degradation could be impacted in strains growing with aeration. Serine degradation is a well-studied process between domains of life (20-35). The RidA Paradigm is a part of one of the serine degradation processes, in which pyruvate is the ketoacid produced. However, there is another serine degradation process that involves other serine deaminases that are Fe-S cluster dependent enzyme, which differs from IlvA being a PLP-dependent enzyme. In the literature, is it known that Fe-S clusters are damage by oxidative stress caused by reactive oxygen species (ROS). ROS attack Fe-S clusters, which inactivate and damage the clusters and the enzymes. There was some merit in thinking that the reason behind the serine sensitivity seen in a *ridA* mutant in shaking, but not static conditions, was because of the serine degradation enzymes that possess a Fe-S clusters being damaged and not being able to degrade exogenous serine that entered the cell (36-41).

In static conditions, where there is less oxygen present in the environment, there should be less damage to Fe-S clusters, meaning that the enzymes (serine deaminases like SdaA and TdcG) should be more active. Our model explains that in shaking conditions in a *ridA* mutant, serine deaminases like SdaA are damaged by ROS due to the presence of oxygen. Without other serine deaminases active to detoxify exogenous serine being transported into the cell, IlvA is primarily responsible for serine deamination. However, with IlvA doing so, more 2AA is generated and the absence of *ridA* does not allow for rapid generation of pyruvate. This could cause 2AA to accumulate, thus attacking PLP-dependent enzymes. In static conditions, there are expected to be fewer ROS, thus not attacking Fe-S cluster dependent serine deaminases like SdaA. In *ridA* mutants when

serine is added in growth media, SdaA can help degrade exogenous serine being pumped into the cell, allowing for the strain to grow. However, this does not alleviate 2AA stress generated by endogenous serine pools; meaning that *ridA* is only able to withstand serine sensitivity.

To test our model, we used a *ridA sdaA* mutant. The strain was used to test if SdaA was needed for a *ridA* mutant to grow in minimal serine media, meaning that SdaA is damaged in shaking conditions and is the reason that the strain cannot grow, exacerbating 2AA stress. A *ridA sdaA* mutant was gown in static conditions with exogenous serine added to minimal glucose media. The stain was unable to grow with serine present unless exogenous isoleucine was added, which allosterically inhibits IlvA from producing 2AA. These results were consistent with our model that damage to SdaA by ROS contributes to lack of growth of *ridA* mutant in the presence of exogenous serine in shaking conditions.

While this helps understanding how the loss of RidA impacts the greater metabolic network, there are other questions that have arisen from this study. Serine degradation pathways are more compromised in shaking conditions compared to static conditions, which could enhance production of 2AA. As was seen in this study, the growth defects of adding exogenous cysteine and Dap were not alleviated by static conditions. However, with data showing that a serine degradation pathway was disrupted by ROS, there could be more biosynthetic pathways that are related to cysteine and Dap disrupted that could add to the sensitivities seen in a *ridA* mutant.

ROS, having such an impact on a *ridA* mutant's ability to degrade serine, is also a point of study. ROS degradation has been a well-studied pathway. In brief, molecular

oxygen (O_2) is reduced first to superoxide (O_2-), then superoxide is reduced to hydrogen peroxide (H_2O_2) by superoxide dismutases (20, 35-42). Hydrogen peroxide is reduced to hydroxide (OH-) by catalase (35-42). Hydroxide is reduced further to water (H_2O). As mentioned, ROS like superoxide, causes damage to Fe-S cluster dependent enzymes. ROS degradation might be compromised in a *ridA* mutant. The superoxide generator paraquat has been used to determine if generation of superoxide impacts an organism. Paraquat is a chemical that can act as an electron donor to molecular oxygen, generating superoxide. Data shows that a *ridA* mutant is more sensitive to paraquat than a wildtype strain (Collier, Christopherson, unpublished data). These data could implicate a possible defect in the ROS degradation pathway. We plan to explore this observation, which could lead to uncovering more about how RidA, rather the loss of RidA, impacts the metabolic network.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The strains used in this study are derivatives of *S. enterica* serovar Typhimurium LT2 and are listed with their respective genotypes in Table 1. Either No-carbon E medium (NCE) or N-carbon/nitrogen E media was used, supplemented with 1 mM $MgSO_4$ (43-44), trace minerals (45), and 11 mM glucose, 22mM glycerol, or 50mM pyruvate was used as minimal medium. 25mM of nitrate was supplemented as an electron acceptor where indicated. Difco nutrient broth (8 g/L) with NaCl (5 g/L) was used as rich (NB) medium. Luria broth was used for experiments involving plasmid isolation. Difco BioTek agar was added (15 g/L) for solid medium. When present in the culture media and unless otherwise stated, the compounds were used

at the following final concentrations: serine, 1mM or 5mM; isoleucine, 0.3mM; glycine, 1mM; cysteine, 1mM; 2,3-diaminopropionate, 0.25mM, thiamine, 100nM. 5mM of L-alanine was used as a nitrogen source where indicated. All chemicals were purchased from Sigma Aldrich (St. Louis, MO)

Quantitation of growth. Experiments employed biological triplicates and each of three cultures, started from single colonies, were grown overnight in NB as inoculum. Cells were pelleted, resuspended in equal volume of saline (0.85% NaCl) and used to inoculate (1:100) 5 mL of NCE minimal media with indicated carbon source and appropriate supplements. Resulting cultures were incubated at 37ºC either statically or with shaking. Final yield cell, determined by density (OD_{650}) after 20-24 hours, was used to quantify growth**.** Significance ($P<0.05$) was determined by conducting a one-way analysis of variance (ANOVA) and Tukey's posttest using GraphPad Prism (Version 9.2). Standard deviation measured by GraphPad Prism (Version 9.2)

Genetic techniques. Strains with multiple mutations were constructed using standard genetic techniques. Methods for transduction, isolation, and identification of phage-free transductants have been described (46-49). Transductional crosses were performed using the high-frequency general transducing mutant of bacteriophage P22 (HT105/1, *int*-201) (50).

Enzyme assays.

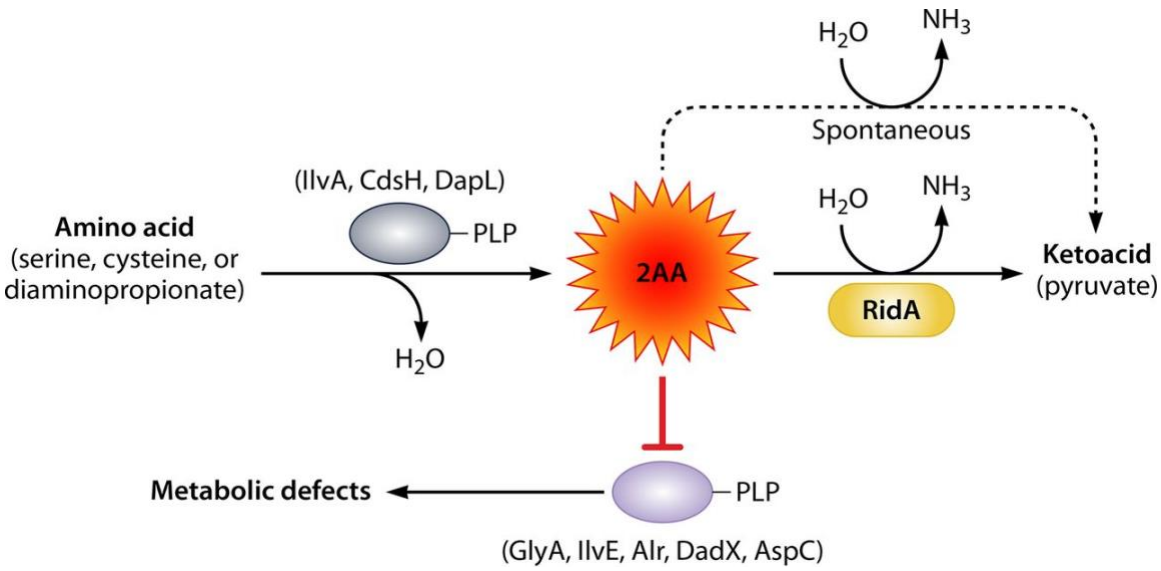
Transaminase B (IlvE). IlvE activity was measured in crude lysates as previously described (7). Briefly, cultures of relevant strains were grown overnight in NB and inoculated 1:100 dilution into NCE glucose media (5mL) with appropriate supplements added as noted in the text. Cultures were grown overnight; cells were pelleted and wash ed with 1X NCE

before being stored at -80C. Cell pellets were thawed and resuspended in 50mM Potassium Phosphate buffer (pH 7.5). Cells were lysed with Constant Systems Limited One Shot (United Kingdom) at 20psi. Cell lysate was clarified by centrifugation at 17000 × *g* for 20 minutes at 4C. Crude lysates were preincubated with mix of PLP, 2-ketoglutarate and buffer for 10 minutes at 37C. Reaction was started with 0.2M isoleucine and after 20 minutes, reaction was terminated by 200uL of 0.3% DNPH (2, 4-dinitrophnylhydrazine). Following derivatization, hydrazone was extracted with 1mL toluene, washed with 500uL 0.5N HCl and treated with 1mL NaOH (1.5N). Absorbance of chromophore layer was measured against 2KMV standards at A540nm. Protein concentrations of crude extracts determined by using the bicinchoninic acid (BCA) assay (Pierce) (51-52).

Threonine and Serine Deaminase Assays. Threonine and Serine Deaminase activity was measured in crude lysates as previously described (7). Briefly, cultures of relevant strains were grown overnight in NB and inoculated 1:100 dilution into NCE glucose media (250mL shaking or 500mL static growth) with appropriate supplements added as noted in the text. Cultures were grown overnight, cells were pelleted and washed with 1X NCE before being stored at -80C. Cell pellets were thawed and resuspended in 50mM Potassium Phosphate buffer (pH 7.5) with 0.4mM DTT. Cells were lysed with Constant Systems Limited One Shot (United Kingdom) at 20psi. Cell lysate was clarified by centrifugation at 17000 × *g* for 20 minutes at 4C. Crude lysates were preincubated with mix of PLP, Tris-HCl pH 8, 5.3% NH_4Cl, 20mDTT, ddH_2O for 5 minutes at 37C. Reaction was started with 400mM threonine or serine and after 20 minutes, reaction was terminated by 3ml of 0.025% DNPH (2, 4-dinitrophnylhydrazine) and 0.9mL of ddH_2O. Following derivatization, hydrazone was treated with 1mL 40% KOH. Absorbance of chromophore

layer was measured against 2KB or Pyruvate standards at A540nm. Protein concentrations of crude extracts determined by using the bicinchoninic acid (BCA) assay (Pierce) (51-52).

Figure 1: The RidA Paradigm of 2AA Stress in *S. enterica*



Amino acids (serine, cysteine or Dap) are converted to the toxic intermediate 2-aminoacrylate (2AA) by certain PLP-dependent enzymes (IlvA, CdsH, DapL). RidA or solvent water can convert 2AA to ketoacids like Pyruvate. In the absence of RidA 2AA accumulates and attacks certain PLP-dependent enzymes, inhibiting activity and potentially compromising growth. Taken from Irons *et al*, 2020.

Table 1. Serine sensitivity of *ridA* mutant is suppressed by static growth

		Shaking[a]			Static[b]		
		Yield in minimal glucose medium with:					
Strain	Relevant Genotype	No addition	Ser	Met	No addition	Ser	Met
DM9404	Wildtype	1.2 ± 0.01	1.2 ± 0.01	1.2 ± 0.1	0.6 ± 0.1	0.6 ± 0.01	0.6 ± 0.01
DM3480	*ridA*	1.2 ± 0.01	0.1 ± 0.01[c]	1.3± 0.1	0.6 ± 0.01	0.5 ± 0.1	0.5 ± 0.01
DM12229	*metE*	0.1 ± 0.01[c]	0.1 ± 0.01[c]	1.2 ± 0.01	0.6 ± 0.1	0.6 ± 0.01	0.6 ± 0.01

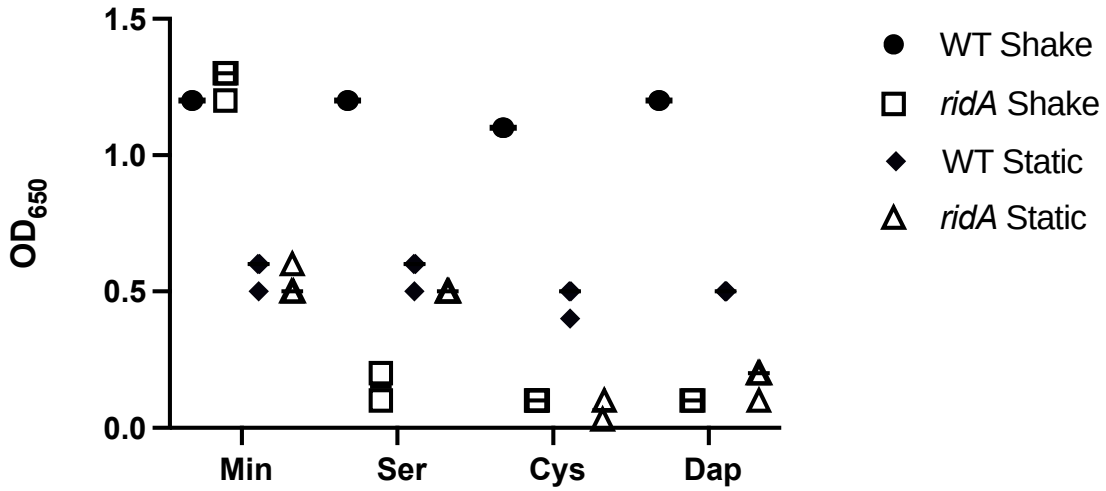
[a] Cultures were incubated with shaking at 37°C

[b] Cultures were incubated without shaking in a 37°C incubator.

[c] Significance ($P<0.05$) was determined by conducting a one-way analysis of variance (ANOVA) and Tukey's posttest using GraphPad Prism (Version 9.2) and is the comparison of growth between wildtype vs *ridA* (*)

Cultures were grown in the indicated medium and condition for 24 hours. Yield was determined as the OD_{650} was taken after 24 hours of growth.

Figure 2: Other generators of 2AA stress are not suppressed when grown statically



Strains were grown in minimal glucose media with cysteine (1mM) or 2, 3-diaminopropionate (0.25mM) present. Cultures were grown at 37C shaking or Static. Experiment completed using biological triplicates. Significance ($P<0.05$) was determined by conducting a one-way analysis of variance (ANOVA) and Tukey's posttest using GraphPad Prism (Version 9.2) and is the comparison of growth between wildtype vs *ridA* (*)

Table 2: Respiration is not required for serine sensitivity of a *ridA* mutant.

		Shaking[a]				Static[b]			
Strains	Relevant genotype	Glucose	Glucose Serine	Glycerol	Glycerol Serine	Glucose	Glucose Serine	Glycerol	Glycerol Serine
DM9404	Wildtype	1.4 ± 0.02	1.4 ± 0.1	1.4 ± 0.01	1.3 ± 0.01	0.7 ± 0.02	0.7 ± 0.02	0.4 ± 0.01	0.5 ± 0.01
DM3480	*ridA*	1.4 ± 0.04	0.1 ± 0.01[c]	1.4 ± 0.01	0.1 ± 0.01[c]	0.6 ± 0.03	0.6 ± 0.01	0.3 ± 0.01	0.3 ± 0.01[c]

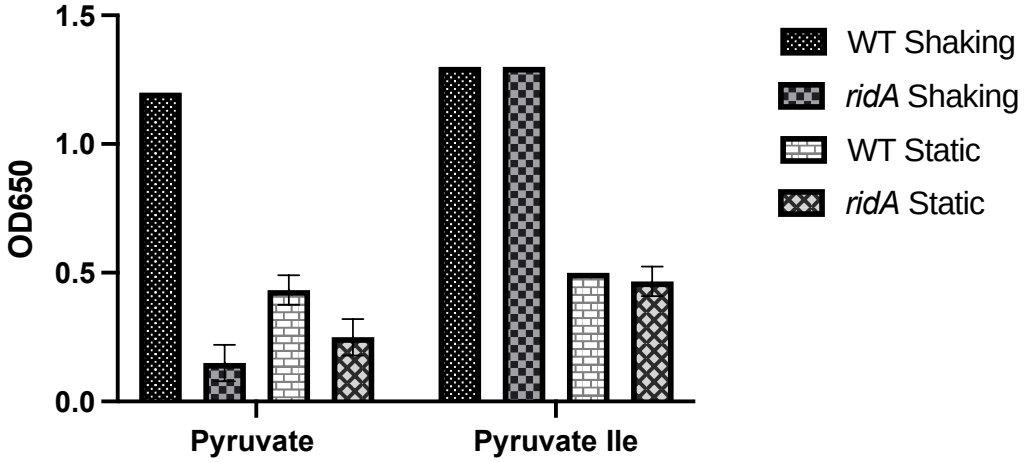
[a] Cultures were incubated with shaking at 37°C

[b] Cultures were incubated without shaking in a 37°C incubator.

[c] Significance ($P<0.05$) was determined by conducting a one-way analysis of variance (ANOVA) and Tukey's posttest using GraphPad Prism (Version 9.2) and is the comparison of growth between wildtype vs *ridA* (*)

Strains were grown in either minimal glucose (11mM) or glycerol (22 mM) and nitrate (25mM) with or without serine (5mM) as indicated and incubated at 37C with or without shaking. Growth yield was determined as the OD_{650} was taken after 24 hours of incubation.

Figure 3: A *ridA* mutant cannot grow in minimal pyruvate medium shaking or static



[a] Cultures were incubated with shaking at 37ºC and without shaking in a 37ºC incubator.

[b] Significance ($P<0.05$) was determined by conducting a one-way analysis of variance (ANOVA) and Tukey's posttest using GraphPad Prism (Version 9.2) and is the comparison of growth between wildtype vs *ridA*

Strains were grown in either minimal pyruvate (50mM) with nitrate (25mM) and was incubated at 37C with or without shaking. Growth yield was determined as the OD_{650} was taken after 24 hours of incubation. Experiment completed using biological triplicates

Table 3: IlvE Activity in *ridA* mutants grown statically is not increased

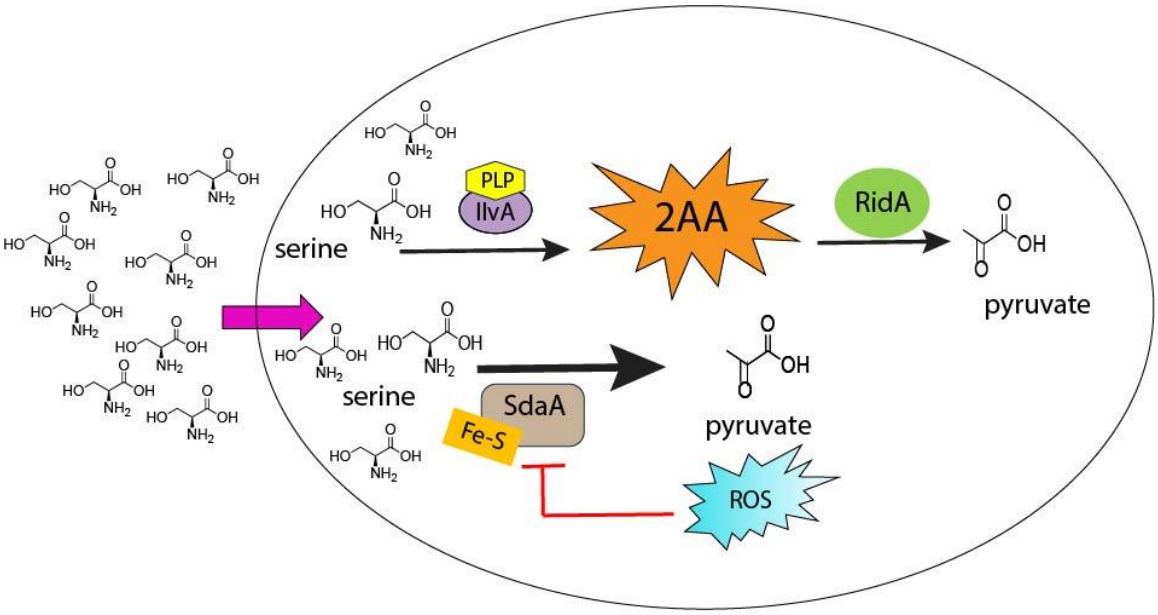
Genotype	Growth Conditions	Addition	2KMV Formation (nmol/mg)	Percentage Difference
Wildtype	**Shaking**	**None**	**472± 11**	**100%**
ridA	**Shaking**	**None**	**314± 8[b]**	**67%**
Wildtype	**Static**	**None**	**376± 1**	**100%**
ridA	**Static**	**None**	**207± 1[b]**	**55%**

[a] Cultures were incubated with shaking at 37ºC and without shaking in a 37ºC incubator.

[b] Significance ($P<0.05$) was determined by conducting a one-way analysis of variance (ANOVA) and Tukey's posttest using GraphPad Prism (Version 9.2) and is the comparison of growth between wildtype vs *ridA*

Strains were grown in minimal glucose media. Cultures were grown to mid or late-log phase. Activity of IlvE from three biological replicates was determined based on 2-ketomethylvalerat (2KMV) formation.

Figure 4: Working Model of SdaA importance in a *ridA* mutant in *S. enterica*.



Aeration compromises SdaA activity, which increases serine available for IlvA and boosts 2AA formation. Serine deaminase SdaA is an [Fe-S] cluster enzyme that deaminates serine without generating 2AA (8). Reactive oxygen species (ROS) attack Fe-S cluster dependent enzymes, in some cases inactivating them. Different levels of 2AA generate different effects, low level prevents growth with pyruvate as carbon source, high level prevents growth by inhibiting serine hydroxymethyl transferase (GlyA) (2).

Table 4: Loss of SdaA worsens serine sensitivity in static conditions.

		Static:		
Strains	Relevant Genotype	No Addition	Ser	Ser Ile
DM3480	*ridA*	0.5± 0.01	0.5± 0.1	0.7± 0.1
DM12933	*sdaA*	0.7± 0.1	0.7± 0.01	0.7± 0.01
DM12931	*ridA sdaA*	0.6± 0.01	0.2± 0.1 [b]	0.8± 0.1

[a] Cultures were incubated without shaking in a 37ºC incubator.

[b] Significance ($P<0.05$) was determined by conducting a one-way analysis of variance (ANOVA) and Tukey's posttest using GraphPad Prism (Version 9.2) and is the comparison of growth between wildtype vs *ridA* (*). Cultures were grown at 37C Static.

1mM Serine and 0.3mM isoleucine.

Table 5: Strain List

Strain Number	Genotype
DM9404	**Wildtype**
DM3480	***ridA3::mudJ***
DM4748	***ilvA595::Tn10***
DM5062	***ridA3::mudJ ilvA595::Tn10***
DM12229	***metE::cat***
DM12931	***ridA3:: mudJ sdaA1::Tn10***
DM12933	***sdaA1::Tn10***
DM14178	***alr::cat***
DM14179	***alr::cat ridA3::mudJ***

All strains were derived from Salmonella enterica serovar Typhimurium LT2 and were obtained from the lab archive or made for this study (46).

MudJ refers to the MudJ1734 insertion element. Tn10d (Tc) refers to the transposition-defective mini-Tn10 (Tn10Δ16Δ17) (45).

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CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSION

The previous chapter described how damage to the serine deaminase SdaA by ROS can impact the ability of a *ridA* mutant in *S. enterica* to grown in minimal serine medium when grown in aerated conditions. This discovery has allowed for the continued progression of knowledge of the RidA Paradigm. Previously stated, RidA is an imine/enamine deaminase that is known to deaminate the reactive intermediate 2-aminoarcylate (2AA). 2AA is generated by certain PLP-dependent enzymes like IlvA, CdsH, and DapL. In the absence in RidA, 2AA can attack and permanently damage certain PLP-dependent enzymes like IlvE, GlyA, and Alr. Generation of 2AA using serine as a substrate via IlvA is the reason why a *ridA* mutant cannot grow in minimal glucose serine medium in normal laboratory shaking conditions.

A *ridA* mutant was able to grow in minimal glucose serine medium in static growth conditions, when no aeration was present. This observation led to the question of the study that was the basis of the thesis described in Chapter 2: Why does the lack of presence of aeration impact a *ridA* mutant's growth in minimal serine medium? A *ridA* mutant grew with and without aeration when glycerol was the carbon source and nitrate provided as an alternative electron acceptor, and the *ridA* mutant was sensitive to serine in minimal glycerol medium when cultures were aerated, ruling out a possibility that serine sensitivity required respiration.

Other generators of 2AA stress in exogenous cysteine (substrate used by CdsH) and 2’3-diaminopropionate (substrate used by DapL) were added to minimal glucose medium to test if there were differences in growth with serine. A *ridA* mutant was not able to grow when exogenous cysteine and Dap was added to growth medium. This showed that other generators of 2AA did not present like serine and IlvA.

2AA stress was not lessened under static conditions. Well-known, the IlvE activity of a *ridA* mutant is decreased compared to a wild-type strain in shaking conditions. IlvE activity of a *ridA* mutant is also decreased in static conditions in comparison to a wildtype strain. A *ridA* mutant is known to not be able to grow utilizing pyruvate as a carbon source, due to endogenous 2AA stress caused by damage to IlvE, thus accumulating pyruvate and an isoleucine requirement. In static conditions, a *ridA* mutant cannot grow in minimal pyruvate medium, unless exogenous isoleucine is added. This data proves that even with serine sensitivity lessened, a *ridA* mutant still has significant 2AA stress.

Serine sensitivity being decreased in less aerated conditions presented a possible model to be tested. Certain serine deaminases have an iron-sulfur cluster (Fe-S) co-factor and Fe-S clusters are known to be damaged by reactive oxygen species (ROS) such has superoxide. It was possible that damage to these serine deaminases impacted the ability for the cell to degrade exogenous serine added to growth medium in shaking conditions. When a *ridA sdaA* mutant was grown in minimal serine medium in static conditions, the strain is unable to grow. This discovery provides some proof that the model could be correct. In shaking conditions, ROS in the environment are generated and can damage Fe-S dependent enzymes like SdaA. The potential damage to SdaA prevents the

detoxification of exogenous serine. In a *ridA* mutant is grown in shaking conditions, and with the damage to SdaA, accumulation of 2AA caused because the lack of detoxification enzymes is the reason behind serine sensitivity.

These new discoveries have added more details to the RidA Paradigm. How the absence of RidA impacts the metabolic network is still being uncovered. The thesis presented herein gives insight this impact. While seemingly a simple phenotype, the finding that a *ridA* mutant was sensitive to exogenous serine has led to over 20 years of studies that have advanced the field. Genetic and biochemical methods are instrumental into the progress being made in these studies. The next section will dissect future directions of the lab research as it pertains to further studies of the RidA Paradigm of 2AA Stress.

FUTURE DIRECTIONS

Presented in Chapter 2, a *ridA sdaA* mutant cannot grow in minimal medium with serine. This observation led to the hypothesis that SdaA, which has an Fe-S cluster that can damaged by reactive oxygen species, cannot degrade serine. This is impacting a *ridA* mutant because serine could be used more by IlvA by default, generating 2AA accumulation. To test this possibility, activity of SdaA can be tested. Experimentally, SdaA activity would be tested from crude extracts from wildtype and *ridA* mutants (or *ilvA* and *ridA ilvA* mutants) grown shaking and statically. The expectation would be that in strains grown in shaking conditions would have low SdaA activity, while strains grown in static conditions would have higher SdaA activity. The higher SdaA activity would indicate less ROS present in the static environments, allowing for SdaA to degrade

serine, alleviating serine sensitivity of *ridA* mutants in static conditions. If the experimental results are the same as predicted, then there is evidence that SdaA activity being lowered in shaking conditions could contribute to the serine sensitivity in *ridA* mutants. If the experimental results are different as predicted, then there could be another serine deaminase with an Fe-S cluster that could be impacted (TdcG or SdaB), all though this is unlikely.

As mentioned in Chapter 1, ROS are generated through their respective degradation pathways. ROS looks to be one of the reasons that a *ridA* mutant cannot degrade exogenous serine. While this was an important discovery, this leads to more questions about how the generation of ROS in *ridA* mutant could be different than a wild-type strain. ROS are degraded through the superoxide radicals degradation pathway Briefly, superoxide is degraded to hydrogen peroxide by enzymes SodA, SodB, and SodC (superoxide dismutase, E.C. 1.15.1) (1-7). Hydrogen peroxide is then degraded to dioxygen by enzymes KatG and KatE (catalase, 1.11.1.6/1.11.1.21) (8-14). In mutants that lack superoxide dismutase and catalase, there are noticeably growth defects due to the absence of these enzyme the degrade generated ROS.

A phenotype seen during our studies showed that a *ridA* mutant was more sensitive to the superoxide generator paraquat than a wildtype strain. Paraquat generates superoxide by being an electron donor for molecular oxygen. This phenotype could indicate that the superoxide degradation process in a *ridA* mutant is disrupted. A pursuit of this could be crucial is understand if the absence of RidA impacts other metabolic processes that have not previously been thought of before. While a *ridA* mutant seems to be or sensitive to superoxide, it would be wise to understand if the strain is sensitive to

other ROS (hydrogen peroxide). Generating *ridA sodA* or *ridA katG* mutants, then growing the strains in minimal glucose medium to observe any growth defects could indicate the importance of these enzymes, telling if the entire or part of the superoxide radical degradation pathway is disrupted.

Overall, this study that is the basis of the thesis described above has opened possibilities for further study of how the metabolic network is disrupted in *S. enterica* and other organisms when *ridA* is deleted. The next directions, which are somewhat still unclear, has at least a narrowed path in discovery of how and why RidA is important for the function of such organisms. The hope is to uncover as much as possible to aid the field towards understanding the impact of small proteins in all domains of life. This thesis has aimed and has accomplished this goal.

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