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Decreased coenzyme A levels in *ridA* mutant strains of *Salmonella enterica* result from inactivated serine hydroxymethyltransferase

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Summary

The RidA/Yer057/UK114 family of proteins is well represented across the domains of life and recent work has defined both an *in vitro* activity and an *in vivo* role for RidA. RidA proteins have enamine deaminase activity, and in their absence the reactive 2-aminoacrylate (2-AA) accumulates and inactivates at least some pyridoxal 5'-phosphate (PLP)-containing enzymes in *Salmonella enterica*. The conservation of RidA suggested that 2-AA was a ubiquitous cellular stressor that was generated in central metabolism. Phenotypically, strains of *S. enterica* that lack RidA accumulated significantly more pyruvate in the growth medium than wild-type strains. Here we dissected this *ridA* mutant phenotype and showed it was an indirect consequence of damage to serine hydroxymethyltransferase (GlyA; E.C. 2.1.2.1). The results here identified a fourth PLP enzyme as a target of enamine stress in *Salmonella*.

Introduction

The RidA/Yer057/UK114 family of proteins is highly conserved, with representative members throughout all domains of life. RidA had enamine deaminase activity *in vitro*, where it accelerated the hydrolysis of three- and four-carbon enamine metabolites generated in the reaction mechanism of PLP-dependent dehydratases. 2-Aminoacrylate (2-AA), the serine derived enamine, is generated in a number of biosynthetic and catabolic reactions *in vivo* (Hillebrand *et al.*, 1979; Schnackerz *et al.*, 1979; Eliot and Kirsch, 2004; Zhao and Liu, 2008), and is known to inhibit a number of PLP-containing enzymes *in vitro* (Flavin and Slaughter, 1969; Relyea *et al.*, 1974; Likos *et al.*, 1982; Badet *et al.*, 1984; Kishore, 1984; Esaki and Walsh, 1986). Despite its reactivity *in vitro*, prior to characterization of RidA, 2-AA was not considered physiologically significant due to its short half-life in aqueous solutions. Recent results showed that the removal of RidA from strains of *Salmonella enterica* resulted in 2-AA-mediated inactivation of PLP-containing enzymes alanine racemases (Alr and DadX) and transaminase B (IlvE) (Flynn and Downs, 2013; Lambrecht *et al.*, 2013). Results from those studies emphasized that the half-life of 2-AA in the cellular environment was long enough to allow irreversible damage of some cellular components. Based on a combination of *in vivo* and *in vitro* results we proposed that at least one role of

RidA family members was to minimize levels of free 2-AA in a cell and prevent damage caused by this reactive metabolite (Lambrecht *et al.*, 2012; 2013). The broad conservation of the RidA family suggests that metabolite stress is an unavoidable consequence of some PLP-dependent chemistries and that the RidA protein family provides one solution to this problem.

Past work identified a variety of phenotypes of *ridA* mutants in *S. enterica* and other organisms (Enos-Berlage *et al.*, 1998; Schmitz and Downs, 2004; Browne *et al.*, 2006; Christopherson *et al.*, 2008; 2012). The identification of a biochemical function for the protein family, and subsequent *in vitro* and *in vivo* results suggested that each phenotype could be attributed to an inactivated PLP-dependent enzyme. Previous results suggested that in the absence of RidA a stressor (e.g. 2-AA) could accumulate and inactivate some percentage of target PLP-dependent enzymes. Thus collectively, the *ridA* mutant phenotypes provided a means to identify metabolite stressors, their endogenous source and their intracellular targets.

This study was initiated to identify the compromised enzyme in a *ridA* mutant that was responsible for the increased accumulation of pyruvate in the growth medium when glucose was sole carbon source. Nutritional and genetic approaches determined that an enzyme in one-carbon metabolism, serine hydroxymethyltransferase, GlyA, was partially inactivated in a *ridA* strain, which indirectly resulted in the accumulation of pyruvate in the medium. Together the data herein expand our understanding of the phenotypic implications of perturbing the metabolic network and identify a fourth target for the 2-AA that accumulates in *ridA* mutant strains of *S. enterica*.

Results and discussion

Ketoacids accumulate in growth media of *ridA* mutant strains

Structural studies performed before the biochemical activity of RidA was defined showed that RidA proteins bind a number of ketoacids (Parsons *et al.*, 2003; Burman *et al.*, 2007). Partially motivated by these results, the growth media of *ridA* mutants were analysed for aberrant ketoacid accumulation. Samples of supernatant were taken periodically during growth of wild type and *ridA* cultures in minimal media with glucose as the carbon source. In each sample, the culture supernatants were treated with dinitrophenolhydrazine to derivatize any monocarboxylic ketoacid and generate stable ketoacid-hydrazones. Total ketoacid-hydrazone concentrations were quantified by measuring absorbance at 443 nm (Friedemann and Haugen, 1943; Dawson *et al.*, 1986). In both wild-type and *ridA* cultures ketoacids accumulated as the cells entered late log phase and disappeared when cells entered stationary phase (Fig. 1A). Significantly, ketoacid accumulation in the *ridA* culture medium was more than eightfold higher than in the wild-type culture. When succinate or gluconate were utilized as the sole carbon source, ketoacids did not accumulate (data not shown) which suggested that flux through Embden–Meyerhof–Parnas glycolysis pathway contributed to the effect.

Hydrazones in the dinitrophenolhydrazine-derivatized supernatants were separated by HPLC and monitored at 380 nm (Fig. 1B). The identities of the precursor ketoacids were

determined by using authentic standards and mass spectral analysis. Pyruvate was the major ketoacid in both supernatants and in the *ridA* culture supernatant, significant ketoisovalerate (KIV) was also detected. These data showed that the absence of RidA resulted in a significant imbalance in the metabolic network around pyruvate.

Mutants lacking RidA accumulate pyruvate due to lowered coenzyme A levels

The activity of transaminase B (IlvE) is reduced in a *ridA* strain (Schmitz and Downs, 2004; Lambrecht *et al.*, 2013), providing a potential explanation for the accumulation of ketoisovalerate noted above (Fig. 2). However, pyruvate accumulation was not an expected outcome of decreased transaminase B activity, suggesting that this phenotype was an uncharacterized consequence of a *ridA* mutation. Pyruvate is utilized by three main enzymes; pyruvate dehydrogenase (PDH), pyruvate formate lyase (PFL) and pyruvate oxidase (POX), none of which are PLP-dependent. When assayed in crude extract, no difference in activity of these enzymes between *ridA* and wild-type strains was detected (data not shown).

The glycolytic conversion of pyruvate to acetyl-coA requires coenzyme A (CoA) as a co-substrate. Radmacher *et al.* showed that mutations in the pantothenate biosynthetic genes *panBC* of *Corynebacterium glutamicum* decreased the intracellular concentration of CoA and resulted in the accumulation of pyruvate (Radmacher *et al.*, 2002). Based on this precedent, pantothenate was added to the medium to raise internal CoA levels and then pyruvate accumulation was measured in a *ridA* strain. Exogenous pantothenate eliminated the majority of pyruvate accumulation by a *ridA* strain (Fig. 3A), suggesting that the pyruvate accumulation resulted from decreased CoA pools. Consistent with this interpretation, total CoA levels were 2.8-fold less in a *ridA* strain than those found in the wild type. Furthermore, exogenous pantothenate restored the CoA levels in a *ridA* strain (Table 1).

Lowered CoA levels in *ridA* mutants are due to a defect in one-carbon metabolism

The data above suggested that pantothenate biosynthesis was compromised in a *ridA* strain, despite the lack of a PLP-dependent enzyme in this pathway. Adding 2-ketopantoate or β -alanine to the medium and monitoring pyruvate accumulation during growth determined which branch of pantothenate biosynthesis (Fig. 2) was compromised (Fig. 3B). Pyruvate did not accumulate when 2-ketopantoate was added, while the addition of β -alanine had no effect. Significantly, 2-ketopantoate is derived from KIV and the data above showed that KIV accumulated in the growth medium of *ridA* mutants. Taken together these results suggested that the enzymatic step catalysed by ketoisovalerate hydroxymethyltransferase (PanB) was compromised in a *ridA* strain. This conclusion was consistent with the finding that exogenous addition of KIV (100 μ M) lowered but did not eliminate pyruvate accumulation (Fig. 3C).

PanB catalyses a reaction that utilizes 5,10-methylenetetrahydrofolate as a co-substrate to formylate KIV and generate 2-ketopantoate. Thus, a limitation for the one-carbon unit carrier 5,10-methylene-tetrahydrofolate could explain the lowered CoA levels detected in a *ridA* strain. To increase 5,10-methylene-tetrahydrofolate levels, exogenous glycine was

added to the growth medium of the *ridA* strain. Degradation of glycine by the inducible glycine cleavage complex generates 5,10-methylene-tetrahydrofolate (Stauffer *et al.*, 1989). Exogenous glycine significantly reduced the pyruvate accumulation in the culture of a *ridA* strain (Fig. 3C), supporting the hypothesis that *ridA* strains were limited for 5,10-methylene-tetrahydrofolate. The exogenous addition of glycine also significantly increased the CoA levels in a *ridA* strain (Table 1). Taken together, these results suggested that under these growth conditions, *ridA* mutants lacked sufficient 5,10-methylene tetrahydrofolate to satisfy the demand for coenzyme A biosynthesis. Further, these data indicated that a defect in one-carbon unit synthesis was responsible for the lowered CoA levels in a *ridA* mutant. Furthermore, the addition of glycine, but not pantothenate, corrected the slight growth defect seen in Fig. 1 (data not shown), suggesting the defect of one-carbon units synthesis has additional effects on cell growth.

***ridA* mutants have lowered serine hydroxymethyltransferase activity**

During growth on glucose *S. enterica* derives one-carbon units from the conversion of serine to glycine *via* the PLP-containing enzyme serine hydroxymethyltransferase (GlyA) (Fig. 2) (Green *et al.*, 1996). When assayed in cell-free extracts, GlyA activity was more than fivefold decreased in *ridA* strain (DM3480) compared with wild type (DM9404) (Table 2). The activity of GlyA was not affected by the addition of pantothenate to the medium, indicating that while pantothenate increased CoA levels, it did so by acting downstream of the GlyA catalysed reaction.

GlyA isolated from a *ridA* strain had reduced specific activity and distinct spectral characteristics

To determine the nature of GlyA inhibition, the enzyme was isolated to > 95% purity from wild-type and *ridA* strains in the presence of PLP cofactor. After isolation, the hydroxymethyltransferase-specific activity of the protein from the *ridA* background was 25% lower than the protein isolated from the wild-type strain (1.47 ± 0.1 and 1.14 ± 0.1 $\mu\text{mol glycine min}^{-1} \text{mg}^{-1}$ for protein isolated from wild type and *ridA* respectively). The decreased specific activity indicated that the inactivated GlyA was at least partially stable through purification, consistent with the presence of a post-translational modification.

The GlyA protein purified from a wild-type strain had different spectral properties than the GlyA protein purified from a strain lacking *RidA*. Enzymes isolated from both strains had an absorbance maximum at 420 nm, which is characteristic of a PLP internal aldimine (Fig. 4A) in the absence of substrate. The similar specific absorbance between the two samples suggested that roughly the same amount of cofactor was bound to the protein in each preparation. In the presence of substrates glycine and tetrahydrofolate, the absorbance spectra of GlyA shifts, with absorbance at 420 nm decreasing and a new peak at 490 nm forming. The later absorbance maximum corresponds to a quinoid species generated when glycine loses an α -proton and forms a carbanion in resonance with the PLP ring (Schirch *et al.*, 1985) (Fig. 5A). As expected, when glycine and tetrahydrofolate were added to the GlyA protein purified from a wild-type strain, the peak at 420 nm decreased with the simultaneous appearance of a peak at 490 nm, indicating the quinoid intermediate had been formed (Fig. 4B). However, when the substrates were added to the enzyme isolated from the

ridA strain, only a partial spectral shift was observed, suggesting the formation of the quinoid species was blocked in a subpopulation of the enzyme (Fig. 4B). A rough quantification, assessed by integrating the area under the curve of absorbance at 490 nm (normalized to the minimum at 470 nm), found the protein isolated from *ridA* had 73% of the absorbance as the protein purified from the wild type (8.80 and 6.46, wild-type and *ridA* background respectively). This ratio correlated with the respective activities of the two enzyme preparations. From these data we concluded that the GlyA protein isolated from a *ridA* strain had a post-translational modification that did not affect cofactor binding but prevented binding of the substrates and/or the abstraction of the α -proton of the bound glycine.

2-AA is thought to inactivate PLP-containing enzymes by one of two mechanisms: (i) 2-AA attacks the internal aldimine of the cofactor (e.g. alanine racemase) (Badet *et al.*, 1984; Esaki and Walsh, 1986) or (ii) 2-AA first forms an external aldimine which is attacked by a nucleophilic residue in the active site to generate a thioester or ester from cysteine or glutamate/aspartate respectively (e.g. IlvE and aspartate decarboxylase) (Tate *et al.*, 1969). Treatment of mammalian GlyA with *o*-fluoroalanine implicated the later route, where a covalent modification was formed by 2-AA on an active-site cysteine residue (Bisswanger, 1981). The crystal structure of GlyA from *Escherichia coli* [PDB 1DFO (Scarsdale *et al.*, 2000)] showed the closest cysteine residue was > 12 Å from the active site. The sole nucleophilic residue in the proximity of the active site in GlyA from *S. enterica* is the highly conserved glutamate 57. Based on this active-site structure, we suggest GlyA is being inactivated by the scheme in Fig. 5B, which is similar to the one described for aspartate decarboxylase (Tate *et al.*, 1969). In this scenario 2-AA forms an external aldimine in the active site and then is attacked by the nucleophilic Glu57. The subsequent rearrangements and hydrolysis result in an esterified glutamate residue and the release of pyridoxamine phosphate. The resulting modification is unstable due to the ester bond, which is readily hydrolysable. Consistently, after the GlyA protein from *ridA* mutant strain was dialysed overnight in 30 mM phosphate buffer (pH 7.2), the specific activity increased and the spectral features became similar to the protein purified from a wild-type strain (data not shown). These results were consistent with an unstable modification and could explain the difficulty detecting different mass spectral characteristics for the protein isolated from *ridA* (data not shown).

Threonine dehydratase activity is involved in decreasing GlyA activity in vivo

Previous studies showed the activity of the biosynthetic enzyme, threonine dehydratase (IlvA), was responsible for several *ridA* mutant phenotypes (Enos-Berlage *et al.*, 1998; Schmitz and Downs, 2004; Christopherson *et al.*, 2012; Lambrecht *et al.*, 2012). Recent research showed that 2-AA generated from serine by IlvA inhibited IlvE *in vitro* (Lambrecht *et al.*, 2013). The activity of IlvA, and thus its deleterious effects in a *ridA* mutant, are prevented by the allosteric inhibitor, isoleucine. Addition of isoleucine to the growth medium of a *ridA* strain, or presence of an IlvA variant (*ilvA3210*) with a lowered specific activity (Christopherson *et al.*, 2008) prevented ketoacid accumulation (Fig. 1C). Also, growth of a *ridA* mutant with exogenous isoleucine increased CoA levels to 80% of those found in a wild-type strain (Table 1) and doubled the activity of GlyA to 40% that of wild

type (Table 2). Taken together these results suggested the serine deaminase activity of IlvA is involved, but not the only source of 2-AA that inhibits GlyA in the absence of RidA.

Conclusions

This study was initiated to explain a *ridA* mutant phenotype in the context of the biochemical activity recently attributed to the protein family. *S. enterica* strains lacking RidA aberrantly accumulated pyruvate in the growth medium. A combination of *in vivo* and *in vitro* approaches found that the PLP-dependent serine hydroxymethyltransferase was at the root of this phenotype. The data showed that decreased activity of GlyA, most likely caused by 2-AA attack, led to decreased 5,10-methylene tetrahydrofolate availability, which resulted in compromised PanB activity. The resulting decrease in pantothenate synthesis lowered the total CoA pool. Ultimately the CoA limitation generated a constraint in the glycolytic breakdown of pyruvate leading to pyruvate accumulation in the growth media.

The finding that serine hydroxymethyltransferase activity was fivefold lower in a *ridA* mutant emphasized the importance of this protein family for maintaining a robust metabolism. In the growth conditions tested, ~ 10% of the total carbon from glucose would flow through this enzyme. An estimated 5% of the carbon in glucose is required to meet the one-carbon demands of *E. coli* growing in minimal media to synthesize purines, histidine, methionine, pantothenate and to methylate DNA and RNA [while another 5% is required to meet the demands for glycine (Matthews, 1996)]. Based on the central role of GlyA, it was somewhat surprising that the notable phenotype was in a distant branch of the metabolic network.

This work increased our knowledge of the PLP enzymes that are inactivated by 2-AA when RidA is absent and emphasized the diverse phenotypes that can be generated by transmission of perturbations in the metabolic network. Thus far threonine dehydratase (IlvA) is the only cellular enzyme demonstrated to be significant in generating 2-AA *in vivo*. The data herein suggest that this enzyme also contributes to the inactivation of GlyA. However, the inability of the allosteric effector isoleucine to completely restore GlyA activity provides evidence that an additional enzyme(s) is contributing to the metabolic stress caused by enamines. The continued study of *ridA* mutant physiology and the effects of 2-AA *in vivo* will provide clarity to the role of the RidA family throughout life. The results reported here and elsewhere show RidA to be an essential partner to maintain integrity of PLP-containing enzyme activity *in vivo*.

Experimental procedures

Bacterial strains, media and chemicals

All strains used in this study are derivatives of *S. enterica* serovar Typhimurium LT2 and are listed with their genotypes in Table 3. Minimal medium was no-carbon E (NCE) supplemented with 1 mM MgSO₄ (Davis *et al.*, 1980) and 11 mM D-glucose. The following supplements were added where specified: ketoisovalerate (100 μM), 2-ketopantoate (100 μM), β-alanine (100 μM), pantothenate (100 μM), glycine (670 μM) and isoleucine (300 μM). Difco nutrient broth (8 g l⁻¹) with NaCl (5 g l⁻¹) was used as a rich medium. Difco

BiTek agar was added (15 g l^{-1}) for solid medium. When required for plasmid maintenance, ampicillin was added to minimal and nutrient media at 15 and 150 mg l^{-1} respectively. Unless noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Molecular biology – construction of JF4

The pTYB2 (New England Biolabs, IMPACT kit) plasmid was digested with XbaI and NheI to excise the multiple cloning site and the gene encoding the self-cleaving intein chitin-affinity tag. This fragment was cloned into a pBAD24 (Guzman *et al.*, 1995) plasmid digested with NheI and PstI to create pJF3. The *glyA* gene was amplified from *S. enterica* LT2 with primers JMF60 (5'-CCCCATATGTGTTAAAGCGTGAAATGAACAT TGC-3') and JMF61 (5'-TTACTCGAGTGCGTAAACCGGGAAG CGT-3') using Herculanase II Polymerase (Agilent Tech.). Following digestion with NdeI and XhoI, the gene fragment was cloned into pJF3 digested with NdeI and XhoI to create pJF4. The final construct was verified by sequencing the ligation junctions.

Ketoacid detection

Cultures were grown in minimal media and aliquots were taken periodically. Cells were removed by centrifugation and 3 ml of the cell-free culture medium was incubated at room temperature for 10 min with a 1 ml solution of 1% 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2 N HCl to selectively extract monocarbonyl-containing α -ketoacids (Friedemann and Haugen, 1943; Raunio, 1966). Next, 4 ml toluene was added and the sample was vortexed at high speed for 30 s. 3.5 ml organic (top) layer was moved to a new tube. Three microlitres of 10% sodium bicarbonate was added and 50 μl aqueous (bottom) phase was transferred to a microtitre plate containing 150 μl 1.5 N NaOH and ketoacids were quantified by absorbance at 443 nm in a Lambda Bio 40 spectrophotometer (Perkin Elmer).

HPLC separation of ketoacids and mass spectral analysis

Ketoacids were extracted as described above. One millilitre of the 10% bicarbonate aqueous phase was spin-dried in a vacufuge (Eppendorf) and resuspended in 200 μl Solvent A (90:10 10 mM ammonium acetate pH 4.0: acetonitrile). Samples were brought to pH 4.0 with 450 μl acetic acid and filtered by centrifugation through 0.45 μm filter (Spin-X). Twenty microlitres of sample was injected onto an LC-20AT Shimadzu HPLC and separated at room temperature on a Luna 5 μm C18 equilibrated in 30% Solvent B (10:90 10 mM ammonium acetate pH 4.0: acetonitrile), 70% Solvent A. Ketoacid-hydrazones were separated with a gradient at 1 ml min^{-1} : 0-10 min 70:30 Solvent A:Solvent B, 10-20 min gradient to 100% Solvent B, 20-28 min 100% Solvent B, 28-30 min gradient to 70:30 Solvent A:B. Ketoacid-hydrazones were detected at 340 nm using a Shimadzu SPD-M20A diode array detector and fractions containing relevant ketoacid-hydrazones were submitted for analysis to the mass spectrometry (MS) facility at the University of Wisconsin-Madison Biotechnology Center where they were analysed by electrospray ionization-mass spectrometry (ESI-MS) in the negative mode. A precursor scan was used to focus on peaks that contained a fragment with a mass of 182, corresponding to the mass of the cleaved

DNPH moiety. Ketoacid-hydrazones separated by HPLC were compared with authentic samples subjected to the same derivatization and extraction methods.

Detection of pyruvate in culture supernatants

To quantify pyruvate, *p*-dimethylbenzaldehyde was used as a derivatizing agent as it does not react with the other ketoacids present (Holtzclaw and Chapman, 1977). Strains to be tested were grown overnight in 1 ml of rich medium by continuous shaking at 37°C, washed with 100 mM NaCl and inoculated (1:8) into minimal media with indicated supplements. Aliquots were taken periodically and optical density at 650 nm was recorded. The cells were removed by centrifugation (1 min at 14.8 K g) and the supernatants were frozen at –80°C for further analysis. To determine the pyruvate concentration in the supernatant the following were added to 100 µl of sample: 375 µl of 5 N KOH and 375 µl of *p*-dimethylaminobenzaldehyde solution (4.9 mg ml^{–1} methanol). The mixture was allowed to react for 30 min at 37°C after which the absorbance was taken at 420 nm. The concentration of pyruvate in the supernatants was determined using a standard curve with various concentrations of sodium pyruvate. To ensure no interfering compounds were being detected in the assay above, an aliquot of supernatant was depleted of pyruvate using lactate dehydrogenase to reduce pyruvate to lactate using NADH. To deplete pyruvate in 100 µl of supernatant, 5 units of lactate dehydrogenase and 1 µmol NADH were added and allowed to react for 1 h. Subsequent analysis showed no absorbance corresponding to interfering compounds.

Determination of total coenzyme A in cells

Total coenzyme A levels were determined using a previously described method (Allred and Guy, 1969). Briefly, strains to be tested were grown overnight in rich media, washed with 100 mM NaCl and inoculated (1:50) into minimal media. Cultures were grown to 0.4 OD₆₅₀, harvested by centrifugation (8000 g for 12 min), and frozen at –80°C for future analysis. Cells were resuspended in phosphate-buffered saline and disrupted by the addition of formic acid to 0.25 N and incubation on ice for 30 min, vortexing periodically. Cell debris was then separated from lysate by centrifugation (14.8 K g) for 10 min. The lysate was then neutralized by the addition of NH₄OH. Aliquots of lysate were treated with dithiothreitol (0.7% final) to facilitate reductive cleavage of CoA thioesters. Quantification of CoA was performed by coupled enzymatic assay, the reactions contained the following per ml: 330 µl of DTT-treated lysate, 250 µmol Tris (pH 7.2), 50 µmol KCl, 15 µmol malate, 6 µmol acetyl-phosphate, 1 µmol NAD⁺, 3.3 U citrate synthase, 15 U malate dehydrogenase and 7.5 U phosphotransacetylase. The rate of NADH formation was determined by monitoring absorbance at 340 nm.

Serine transhydroxymethylase activity

For activity determination in crude extract, strains were grown in rich media overnight, cells were pelleted and resuspended in NaCl. A culture (1:50 inoculum) was grown in minimal medium to 0.4 OD₆₅₀, cells were harvested by centrifugation (8000 g for 12 min and frozen at –80°C for future analysis. Cell pellets were resuspended in 100 mM potassium phosphate buffer (pH 7.3) with 1 mM EDTA and disrupted by sonication. Cell debris was removed by

centrifugation (14.8 K g) for 10 min. Activity was assayed by modifying a described protocol (Schirch *et al.*, 1985). Each 1 ml assay included: 30 μ l clarified cell lysate (or 1.5 μ g of purified protein), 100 μ mol potassium phosphate (pH 7.2), 0.4 μ mol tetrahydrofolate, 4 nmol pyridoxal 5'-phosphate, 20 μ g FOLD [purified from ASKA collection (Kitagawa *et al.*, 2005)] and 1 μ mol serine. Absorbance was monitored at 340 nm to follow NADPH formation. Glycine production rates were calculated using the extinction coefficient for NADPH at neutral pH (6.22 mM⁻¹ cm⁻¹). Protein concentrations were determined using 660 nm Protein Assay (Thermo Scientific) and bovine serum albumin as a reference.

Serine hydroxymethyltransferase purification

Overnight cultures (50 ml) of strain DM14171 or DM14172 were used to inoculate 2 l of minimal media. Cultures were grown with shaking at 37°C until they reached an OD₆₅₀ of 0.5. At that point arabinose was added to 0.2% final concentration (w/v) to induce *glyA* expression. Cells were harvested by centrifugation (15 min, 9000 g) when OD₆₅₀ was between 2 and 2.5 and the resulting cell pellets were frozen at -80°C. Pellets were resuspended in 20 mM HEPES, 100 mM sodium chloride buffer (pH 8.5), 5 mM EDTA, 5 mM benzamidine and 10 μ M PLP. Cells were broken with a French Pressure cell (2 passes at 1500 psi). After clarification by centrifugation (45 min at 48 K g), the supernatant was applied to chitin resin (column volume 2 ml) and protein purification proceeded per manufacturer's instructions (New England Biolabs, IMPACT). After removal from resin, the protein was concentrated and flash frozen after the addition of glycerol to 10%. PLP (10 μ M) was provided in all buffers.

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Fig. 1.

Ketoacids accumulate in the culture medium of *ridA* mutants.

A. DM9404 (wild type) (open symbols) and DM3480 (*ridA* mutant) strains (filled symbols) were grown in glucose medium and growth was measured as optical density at 650 nm (circles). Monocarbonyl-containing α -ketoacids were detected spectrophotometrically at 443 nm after derivatization and extraction (squares). Data represent two independent cultures.

B. Derivatized α -ketoacids from the culture medium of wild type (black) and a *ridA* mutant (grey) were separated by HPLC and monitored by absorbance at 380 nm. Ketoacids were identified using derivatized standards of authentic ketoacids and by mass spectral analysis. The hydrazone of pyruvate appeared as two separate peaks at 17 and 20 min and was attributed to *syn-anti* isomerization of the pyruvate hydrazone.

C. DM10009 (*ilvA3210 ridA*) was grown in glucose medium (open symbols) while DM10010 (*ridA*) was grown in glucose minimal medium with 0.3 mM isoleucine (closed symbols). Growth of these representative strains was measured as optical density at 650 nm (diamonds) and monocarbonyl-containing α -ketoacids were extracted and quantified spectrophotometrically at 443 nm (triangles). Data points were averaged from two independent cultures.

Fig. 2.

Metabolic pathways in *Salmonella enterica* relevant to pyruvate accumulation. Pathways involved in this study are schematically illustrated, with both their connections and role in metabolism highlighted. Key enzymes are named by the appropriate gene product and are located by the reaction(s) they catalyse. Abbreviations: PDH, pyruvate dehydrogenase; Gcv, glycine cleavage complex; GlyA, serine hydroxymethyltransferase; PanB, ketoisovalerate hydroxymethyltransferase; PanE, ketopantoate reductase; PanC, pantothenate synthetase; IlvE, transaminase B.

Fig. 3. *ridA* mutants accumulate pyruvate in the medium. Shown are the results of representative experiments monitoring pyruvate concentrations over time throughout growth of wild type (DM9404) and *ridA* mutant (DM3480) as a function of optical density of the cultures. Growth media was minimal glucose with indicated additions.

Fig. 4.

Spectral characteristics of GlyA from different backgrounds. GlyA protein was isolated from wild-type and *ridA* mutant strains and the visible spectral characteristics were noted. A. GlyA isolated from wild-type (—) and *ridA* mutant (---) backgrounds in 100 mM potassium phosphate at pH 7.2. B. Spectra of the same samples after the addition of glycine (20 mM) and tetrahydrofolate (0.18 mM).

Fig. 5.

Active-site configuration and proposed modification scheme of GlyA. A. Native serine hydroxymethyltransferase with internal aldimine to the PLP cofactor absorbs at 420 nm. After the addition of glycine and tetrahydrofolate, the glycine and PLP form an external aldimine. The subsequent extraction of an α -proton of glycine generates a species that absorbs at 490 nm. B. An inactivation scheme modelled after the one proposed for aspartate decarboxylase (Relyea *et al.*, 1974) is depicted. In this scenario 2-aminoacrylate forms external aldimine with the active-site PLP, which is then attacked by nucleophilic glutamate residue. Subsequently, the cofactor can be released as pyridoxamine phosphate and glutamate is esterified to the pyruvoyl moiety after hydrolysis.

Table 1

Total CoA levels are decreased in *ridA* strain.

Strain	Genotype	Addition	TotalCoA (nmol CoA mg ⁻¹ dry weight)	% wild type
DM9404	Wild type	None	2.02 ± 0.06	100
DM3480	<i>ridA</i>	None	0.68 ± 0.06	34
DM3480	<i>ridA</i>	Pantothenate	2.12 ± 0.18	105
DM3480	<i>ridA</i>	Glycine	1.68 ± 0.13	83
DM3480	<i>ridA</i>	Isoleucine	1.78 ± 0.16	88

Total CoA was measured in cell pellets of the indicated strains. Strains were grown using glucose minimal media with the indicated additions. Growth and CoA quantification are described in *Experimental procedures*. Data given are the mean and standard deviation of experiments performed in biological duplicate with technical triplicates.

Table 2

Serine hydroxymethyltransferase activity is low in *ridA* strains.

Strain	Genotype	Addition	Activity (nmol glycine min ⁻¹ mg ⁻¹)	% Activity
DM9404	Wild type	None	27.9 ± 4.9	100
DM3480	<i>ridA</i>	None	4.8 ± 1.8	17
DM3480	<i>ridA</i>	Pantothenate	5.7 ± 2.7	20
DM3480	<i>ridA</i>	Isoleucine	11.3 ± 1.2	40

Table 3

Bacterial strains.

Strain	Relevant genotype
DM3480	<i>ridA3</i> ::MudJ
DM9404	Wild type
DM10009	<i>ilvA3210 ridA3</i> ::MudJ
DM10010	<i>ridA3</i> ::MudJ
DM14171	Wild type/pJF4
DM14172	<i>ridA3</i> ::MudJ/pJF4

All strains were either part of the laboratory stock or generated in this study. MudJ refers to the Mud1734 transposon (Castilho *et al.*, 1984). Plasmid pJF4 is a derivative of pBAD24 (Guzman *et al.*, 1995) that expresses *glyA* and is described further in *Experimental procedures*.