Salmonella exploits host- and bacterial-derived β-alanine for replication inside host macrophages

Shuai Ma[1,†], Bin Yang[1,†], Yuyang Sun[1], Xinyue Wang[1], Houliang Guo[1], Ruiying Liu[1], Ting Ye[1], Chenbo Kang[1], Jingnan Chen[1], Lingyan Jiang[1,*]

[1]*National Key Laboratory of Intelligent Tracking and Forecasting for Infectious Diseases, TEDA Institute of Biological Sciences and Biotechnology, Nankai University, Tianjin 300457, China.*

[†] Shuai Ma and Bin Yang contributed equally to this work.

[*]Correspondence and requests for materials should be addressed to Lingyan Jiang (email: jianglingyan@nankai.edu.cn).

Abstract

Salmonella is a major foodborne pathogen that can effectively replicate inside host macrophages to establish life-threatening systemic infections. *Salmonella* must utilize diverse nutrients for growth in nutrient-poor macrophages, but which nutrients are required for intracellular *Salmonella* growth is largely unknown. Here, we found that either acquisition from the host or de novo synthesis of a nonprotein amino acid, β-alanine, is critical for *Salmonella* replication inside macrophages. The concentration of β-alanine is decreased in *Salmonella*-infected macrophages, while the addition of exogenous β-alanine enhances *Salmonella* replication in macrophages, suggesting that *Salmonella* can uptake host-derived β-alanine for intracellular growth. Moreover, the expression of *panD*, the rate-limiting gene required for β-alanine synthesis in *Salmonella*, is upregulated when *Salmonella* enters macrophages. Mutation of *panD* impaired *Salmonella* replication in macrophages and colonization in the mouse liver and spleen, indicating that de novo synthesis of β-alanine is essential for intracellular *Salmonella* growth and systemic infection. Additionally, we revealed that β-alanine influences *Salmonella* intracellular replication and *in vivo* virulence by increasing expression of the zinc transporter genes *znuABC*, which in turn facilitates the uptake of the essential micronutrient zinc by *Salmonella*. Taken together, these findings highlight the important role of β-alanine in the intracellular replication and virulence of *Salmonella*, and *panD* is a promising target for controlling systemic *Salmonella* infection.

Keywords: *Salmonella*, replication in macrophage, virulence, β-alanine, zinc

uptake

Introduction

Salmonella is a major foodborne pathogen worldwide that can cause self-limiting gastroenteritis or life-threatening systemic disease in a wide range of animals (Fàbrega & Vila, 2013; Ohl & Miller, 2001). *Salmonella* infection remains a significant global public health concern. An estimated 93.8 million cases of gastroenteritis and 27 million cases of systemic diseases caused by *Salmonella* species occur annually worldwide, with 355,000 deaths (Kim et al., 2019; Majowicz et al., 2010). The ability to survival and replicate in host macrophages is a key determinant for *Salmonella* to induce systemic infection (Fields, Swanson, Haidaris, & Heffron, 1986; LaRock, Chaudhary, & Miller, 2015; Leung & Finlay, 1991). After internalization by macrophages, *Salmonella* delivers a set of more than 30 effector proteins to the macrophage cytoplasm, mainly through a type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island-2 (SPI-2) (Pillay et al., 2023). SPI-2 effectors manipulate diverse cellular processes to promote the formation of a membrane-bound compartment, termed the *Salmonella*-containing vacuole (SCV), a niche where *Salmonella* resides and grows (Castanheira & García-Del Portillo, 2017; Rosenberg, Riquelme, Prince, & Avraham, 2022; Steeb et al., 2013). SCV protects *Salmonella* from contact with antimicrobial agents in macrophages (Figueira & Holden, 2012; Li et al., 2023).

As the SCV of macrophages is a nutrient-poor environment, to effectively replicate in the SCV, *Salmonella* needs to acquire a wide range of host nutrients or host-derived metabolites and synthesize metabolites de novo that cannot be

sufficiently accessed from the host (Dandekar et al., 2014; Röder, Felgner, & Hensel, 2021; Tuli & Sharma, 2019). Nutrients/metabolites are used by intracellular *Salmonella* either as carbon sources to generate energy or for the synthesis of fatty acids and proteins (Dandekar et al., 2014; Steeb et al., 2013). Moreover, several metabolites were found to be employed by *Salmonella* as environmental cues to induce the expression of virulence genes (Jiang et al., 2021; X. Wang et al., 2023). In recent years, an increasing number of studies have focused on the intracellular nutrition of *Salmonella*; however, the nutrients that are required for *Salmonella* replication in macrophages remain largely unknown.

β-Alanine, also known as 3-aminopropionic acid (3-AP), is the only naturally occurring β-type amino acid and is found in all living organisms. β-Alanine can be synthesized de novo by bacteria, fungi, and plants, whereas animals need to obtain it from food or generate it via the catabolism of cytosine and uracil. In bacteria, β-alanine is synthesized *via* the decarboxylation of L-aspartate, a reaction catalyzed by L-aspartate decarboxylase (PanD) (Begley, Kinsland, & Strauss, 2001; Schmitzberger, Smith, Abell, & Blundell, 2003). The *panD* gene is conserved among most bacteria. Although β-alanine is a nonprotein amino acid that is not incorporated into proteins, it has important physiological functions in the metabolism of organisms (Yuan et al., 2022). First, β-alanine forms a part of pantothenate (vitamin B5), which is the key precursor for the biosynthesis of coenzyme A (CoA) (Webb, Smith, & Abell, 2004; White, Gunyuzlu, & Toyn, 2001). CoA is an essential cofactor involved in many metabolic pathways, including the synthesis and degradation of fatty acids,

pyruvate oxidation through the tricarboxylic acid (TCA) cycle, and the production of secondary metabolites (Davaapil, Tsuchiya, & Gout, 2014; Gout, 2019; Sibon & Strauss, 2016; Theodoulou, Sibon, Jackowski, & Gout, 2014). Second, β-alanine is a limiting precursor of carnosine, a nonenzymatic free radical scavenger and a natural antioxidant, with anti-inflammatory and neuroprotective effects in animals (Boldyrev, Aldini, & Derave, 2013; Hoffman, Varanoske, & Stout, 2018). In the past 15 years, β-alanine has become one of the most commonly used sports supplements worldwide (Bellinger, 2014; Hoffman et al., 2018; Huerta Ojeda, Tapia Cerda, Poblete Salvatierra, Barahona-Fuentes, & Jorquera Aguilera, 2020). Although both *Salmonella* and host cells are capable of producing β-alanine, whether β-alanine contributes to the pathogenicity and intracellular growth of *Salmonella* remains unknown.

In this work, using targeted metabolic profiling, *in vitro* and *in vivo* infection assays, and many other molecular techniques, we demonstrated that the utilization of β-alanine is essential for *Salmonella* replication in host macrophages and virulence in mice. *Salmonella* acquires β-alanine both via the uptake of β-alanine from host macrophages and the de novo synthesis of β-alanine. Further investigation revealed the molecular mechanism underlying the contribution of β-alanine to *Salmonella* intracellular replication and pathogenicity, wherein β-alanine promotes the expression of zinc transporter genes to facilitate the uptake of the essential micronutrient zinc by intracellular *Salmonella*, therefore promoting *Salmonella* replication in macrophages and subsequent systemic infection. Taken together, these findings demonstrate a correlation between *Salmonella* β-alanine utilization and zinc uptake during

intracellular infection and provide new insights into the intracellular nutrition of *Salmonella*. The rate-limiting gene (*panD*) in the β-alanine synthesis pathway of *Salmonella* might be a future target for the prevention and treatment of this pathogen.

Results

Host-derived β-alanine promotes *Salmonella* replication inside macrophages

To explore changes in the levels of different amino acids inside macrophages upon *Salmonella* infection, we performed targeted metabolomics analysis of mouse RAW264.7 macrophages that were mock-infected or infected with wild-type *Salmonella* (*Salmonella enterica* serovar Typhimurium ATCC 14028s, STM) for 8 h using liquid chromatography-tandem mass spectrometry (LC–MS/MS) (Figure 1A). Principal component analysis (PCA) demonstrated a clear separation between the mock- and *Salmonella*-infected groups (Figure 1B). A total of 26 free amino acids were analyzed, and 8 showed significant differences in abundance between the two groups (VIP > 1 and $P < 0.05$; fold change > 1.5 or < 0.667) (Figure 1C). Compared with those in the mock-infected group, the concentrations of 3 amino acids (L-hydroxyproline, L-citrulline and L-cysteine) were upregulated, and 5 amino acids (L-asparagine, L-serine, L-aspartate, β-alanine and γ-aminobutyric acid) were downregulated in the *Salmonella*-infected group (Figure 1C). Consistent with previous findings, intracellular serine concentrations were downregulated due to the reprogramming of macrophage glucose metabolism during *Salmonella* infection (Jiang et al., 2021). *Salmonella* can use host-derived aspartate and asparagine for growth in macrophages (Popp et al., 2015); therefore, the decrease in intracellular aspartate and asparagine upon *Salmonella* infection is likely due to their utilization by bacteria. Interestingly, β-alanine concentrations were also downregulated in the *Salmonella*-infected group (Figure 1C, D), suggesting that intracellular *Salmonella*

may use host-derived β-alanine for growth.

To investigate whether host-derived β-alanine can promote intracellular *Salmonella* replication, we added an additional 1 mM β-alanine (Schneider, Krämer, & Burkovski, 2004) to the culture medium (RPMI) of RAW264.7 cells and then infected them with *Salmonella* to test the influence of β-alanine addition on the ability of *Salmonella* to replicate in macrophages. The results showed that the replication of *Salmonella* in RAW264.7 cells significantly ($P < 0.01$) increased with the addition of β-alanine (Figure 1E), suggesting that host-derived β-alanine promotes *Salmonella* replication inside macrophages. We then investigated whether β-alanine-mediated *Salmonella* growth promotion is due to the changes in antimicrobial activity of the macrophages, *via* quantitative real-time PCR (qRT–PCR) assays and flow cytometry analysis. We observed that the addition of 1 mM β-alanine did not influence the expression of pro- (*Il1b*, *Tnf*) and anti-inflammatory genes (*Il10*, *Il4ra*) of *Salmonella*-infected RAW264.7 cells (Figure 1F), and did not affect the percentage of pro-inflammatory M1 macrophages (CD86+) and anti-inflammatory M2 macrophages (CD163+) during *Salmonella* infection (Figure 1G), implying that the addition of β-alanine to macrophages does not change their immune response. Combining these results, we can further infer that *Salmonella* use host-derived β-alanine for intracellular growth.

Direct validation of *Salmonella* using host-derived β-alanine for intracellular growth requires a mutant that has a defect in β-alanine uptake. *Escherichia coli* uptakes β-alanine via the transporter protein CycA (Schneider et al., 2004). However,

the *Salmonella* Δ*cycA* mutant was able to use β-alanine as the sole carbon source for growth in minimal medium (Supplementary Figure 1A), indicating that CycA is not a transporter for β-alanine in *Salmonella* and that other unknown β-alanine import mechanisms are involved. Consistent with these results, mutation of *cycA* did not influence the replication of *Salmonella* in RAW264.7 cells (Supplementary Figure 1B) or colonization in mouse systemic tissues (liver and spleen; Supplementary Figure 1C).

De novo β-alanine synthesis is critical for *Salmonella* replication inside macrophages

Salmonella can de novo synthesize β-alanine *via* the decarboxylation of L-aspartate, which is catalyzed by L-aspartate decarboxylase (PanD) (Figure 2A) and is reportedly the rate-limiting step of β-alanine generation (Begley et al., 2001; Schmitzberger et al., 2003). To further assess the role of β-alanine in *Salmonella* intracellular replication, we analyzed the expression level of the *Salmonella panD* gene in macrophages and the impact of *panD* mutation on the ability of *Salmonella* to replicate in macrophages. Quantitative real-time PCR (qRT–PCR) assays revealed that the expression level of *panD* was significantly ($P < 0.01$) greater in RAW264.7 cells than in RPMI-1640 medium (Figure 2B). Increased expression of *panD* was also observed in N-minimal medium, a widely used medium that mimics the conditions inside macrophages, as revealed by qRT–PCR and bioluminescent reporter assays (Figure 2C, D). These results demonstrate that *panD* expression is enhanced during

Salmonella growth inside macrophages, suggesting a relationship between *panD* expression and intracellular *Salmonella* growth.

We then constructed the *panD* mutant strain Δ*panD* and compared the replication ability of the Δ*panD* strain and the wild-type (WT) strain in RAW264.7 cells. Gentamicin protection assays showed that the replication of Δ*panD* in RAW264.7 cells decreased 2.4-fold at 20 h postinfection compared with that of the WT strain ($P < 0.01$), while complementation of Δ*panD* with the *panD* gene restored the replication ability of the mutant strain in RAW264.7 cells (Figure 2E). Immunofluorescence analysis revealed that the number of Δ*panD* in each infected RAW264.7 cell was comparable to that of the WT strain at the initial infection stage (2 h), but at 20 h postinfection, the number of Δ*panD* in each infected RAW264.7 cell was significantly ($P < 0.0001$) lower than that of the WT strain (Figure 2F, G). These results indicate that *panD* contributes to *Salmonella* replication in macrophages. The growth rates of Δ*panD* in LB medium and RPMI medium resembled those of the WT (Supplementary Figure 2A, B), indicating that the impaired intracellular replication ability of the mutant was not due to a growth defect. Moreover, the replication defect of Δ*panD* in RAW264.7 cells was relieved by the addition of 1 mM β-alanine to the RPMI medium (Figure 2H). These data collectively suggest that β-alanine synthesis is critical for *Salmonella* replication inside macrophages.

De novo β-alanine synthesis is critical for systemic *Salmonella* infection in mice

As replication in macrophages is a key determinant of systemic *Salmonella*

infection, we reasoned that β-alanine synthesis also influences *Salmonella* virulence *in vivo*. BALB/c mice were infected by i.p. injection of 5,000 CFU of WT, Δ*panD*, or the complemented strain c*panD*. The survival rate, body weight, bacterial burden in the liver and spleen, and liver histopathological alterations of the infected mice were measured (Figure 3A). The WT-infected mice exhibited high lethality and marked loss of body weight within 5 days, and all mice died within 9 days of infection (Figure 3B, C). In contrast, the Δ*panD*-infected mice displayed significantly improved survival rates and body weights, and no mice died within the 10-day surveillance period (Figure 3B, C). Consistent with these results, the bacterial burden in the liver and spleen of the Δ*panD*-infected mice was significantly decreased, and the body weight was significantly increased compared with that of the WT-infected mice on day 3 postinfection (Figure 3D). Complementation of Δ*panD* with *panD* significantly decreased the survival rate and body weight of infected mice but significantly increased the bacterial burden in the liver and spleen of the infected mice (Figure 3B-D). Furthermore, H&E staining revealed increased aggregation of inflammatory cells and pyknosis in the livers of the WT-infected mice on day 5 postinfection, while these histopathological alterations were obviously reduced in the livers of Δ*panD*-infected mice (Figure 3E). Taken together, these results reveal that β-alanine synthesis is critical for systemic *Salmonella* infection in mice.

β-Alanine is involved in the regulation of several metabolic pathways in *Salmonella*

To explore the mechanism(s) associated with β-alanine-mediated promotion of *Salmonella* replication in macrophages and *in vivo* virulence, we performed RNA sequencing (RNA-seq) to reveal the differences in gene transcripts between *Salmonella* WT and Δ*panD* strains grown in N-minimal medium. PCA plot of the global transcriptomic profiles clearly demonstrated separation between the WT and Δ*panD* strains (Figure 4A). Remarkable transcriptional changes were observed due to the mutation of *panD*. Compared with those in the WT strain, 1379 genes were differentially expressed in the Δ*panD* strain, with 561 upregulated genes and 618 downregulated genes (fold change ≥ 2 and P value < 0.05; Figure 4B). Gene Ontology (GO) enrichment analysis revealed that the differentially expressed genes (DEGs) were mainly involved in the metabolism and biosynthesis of several amino acids (including arginine, leucine, histidine, and branched amino acids), carboxylic acid metabolism, small molecule biosynthesis, and aerobic respiration (Figure 4C). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis also revealed a high frequency of terms related to metabolism, including amino acid metabolism, lipid metabolism, carbohydrate metabolism, energy metabolism, and nucleotide metabolism (Figure 4D). These data collectively indicate that β-alanine is involved in the regulation of a series of metabolic pathways in *Salmonella*.

Further analysis of the downregulated DEGs (activated by PanD) revealed that mutation of *panD* decreased the expression of genes involved in 7 pathways that are

associated with the virulence of *Salmonella* or other bacterial pathogens, including methionine metabolism, fatty acid β-oxidation, histidine biosynthesis, and the transport of zinc, galactose, potassium, and polyamine (Figure 4E). Zinc and potassium uptake are associated with the virulence of *Salmonella* (zinc acquisition promotes *Salmonella* Typhimurium virulence in mice, and potassium acquisition promotes *Salmonella* Enteritidis virulence in chickens) (Ammendola et al., 2007; Battistoni, Ammendola, Chiancone, & Ilari, 2017; Ilari et al., 2016; Liu et al., 2013), while the remaining 5 pathways are involved in the pathogenicity of other pathogens (Basavanna et al., 2013; de Paiva et al., 2016; Feldman et al., 2016; Lauriano et al., 2004; Martínez-Guitián et al., 2019). In addition, the expression of the LysR-type transcriptional regulator LeuO, which activates the expression of the *leuABCD* leucine synthesis operon and numerous virulence genes in *Salmonella* Typhimurium (Dillon et al., 2012; Guadarrama, Villaseñor, & Calva, 2014; Hernández-Lucas et al., 2008), was also downregulated in the Δ*panD* strain (Figure 4E). In line with the decreased expression of *leuO*, the expression of *leuABCD* was downregulated in the Δ*panD* strain (Figure 4E).

We selected 16 downregulated DEGs (including the regulatory gene *leuO* and genes from the above 7 pathways) for qRT–PCR analysis. The results showed that the expression of all 16 genes significantly ($P < 0.05$) decreased in the Δ*panD* mutant compared with the WT strain (Figure 4F), and complementation of Δ*panD* with *panD* restored the gene expression to the WT level (Figure 4F), thus confirming the positive regulation of these pathways and LeuO by β-alanine.

Although SPI-2 gene expression is essential for *Salmonella* replication in macrophages and systemic infection, the expression of SPI-2 genes was not influenced by *panD* mutation (Supplementary Figure 3A). In addition, the gene expression of 4 other virulence-associated pathogenicity islands, namely, SPI-1, SPI-3, SPI-4, and SPI-5, was not influenced by *panD* mutation (Supplementary Figure 3B, C).

Taken together, these data suggest that β-alanine might promote *Salmonella* intracellular replication and virulence by activating virulence-associated pathway(s) or activating the virulence-associated regulator LeuO, rather than by activating the expression of virulence genes encoded within pathogenicity islands.

β-Alanine promotes *Salmonella* virulence *in vivo* by increasing the expression of zinc transporter genes

Next, we inactivated the 7 downregulated pathways mentioned above, as well as the regulatory gene *leuO* in *Salmonella*, to uncover the mechanism(s) by which β-alanine promotes *Salmonella* virulence *in vivo*. Mouse infection assays revealed that mutations in *fadAB*, *metR*, *hisABCDFGHL*, *kdpABC*, *mglABC*, and *potFGHI*, which are associated with fatty acid β-oxidation, methionine metabolism, histidine biosynthesis, potassium uptake, galactose uptake, and polyamine uptake, respectively, did not influence *Salmonella* colonization in the mouse liver or spleen (Figure 5A, B) or the body weight of infected mice (Figure 5C). Interestingly, although LeuO has been reported to be associated with the regulation of a diverse set of virulence factors

(Dillon et al., 2012; Guadarrama et al., 2014), mutation of the regulatory gene *leuO* did not influence *Salmonella* colonization in the mouse liver or spleen (Figure 5A, B) or the body weight of infected mice (Figure 5C). In contrast, mutation of the zinc transporter gene *znuA* significantly decreased *Salmonella* colonization in the mouse liver and spleen (Figure 5D, left and middle panels); this result is consistent with previous studies (Ammendola et al., 2007; Battistoni et al., 2017; Ilari et al., 2016). Accordingly, the body weight of Δ*znuA*-infected mice was significantly ($P < 0.001$) greater than that of the WT-infected mice (Figure 5D, right panel). These results indicate that β-alanine might promote *Salmonella* virulence *in vivo* by promoting zinc uptake.

To test this hypothesis, we constructed a double mutant, Δ*panD*Δ*znuA*, and compared colonization of the mouse liver and spleen of the double mutant to that of the single mutant, Δ*znuA*. The results showed that colonization of the liver and spleen of infected mice by Δ*panD*Δ*znuA* was significantly lower than that of infected mice colonized by Δ*znuA* (Figure 5D, left and middle panels). In agreement with these results, the body weight of Δ*panD*Δ*znuA*-infected mice was greater than that of Δ*znuA*-infected mice (Figure 5D, right panel), suggesting that the contribution of *panD* to the virulence of *Salmonella* is partially dependent on *znuA*.

Collectively, these data indicate that β-alanine promotes *in vivo* virulence of *Salmonella* partially by increasing the expression of zinc transporter genes.

β-Alanine promotes *Salmonella* replication within macrophages by increasing the expression of zinc transporter genes

To determine whether β-alanine influences *Salmonella* intracellular replication by acting on zinc transporters, we analyzed the ability of Δ*znuA* and Δ*panD*Δ*znuA* to replicate in RAW264.7 macrophages *via* gentamicin protection assays. The results showed that the replication of Δ*panD*Δ*znuA* in RAW264.7 cells was significantly reduced compared with that of the single mutant Δ*znuA* (Figure 6A), implying that the contribution of *panD* to the intracellular replication of *Salmonella* is partially dependent on *znuA*. The addition of 100 μM Zn^{2+} to RPMI medium increased the replication of Δ*panD* in RAW264.7 cells (Figure 6B), while the addition of 1 mM β-alanine to RPMI medium did not increase the replication of Δ*znuA* (Figure 6C), suggesting that the impaired replication due to the decrease in β-alanine can be relieved by zinc supplementation. Taken together, these data indicate that β-alanine promotes *Salmonella* replication within macrophages by increasing the expression of zinc transporter genes.

Discussion

Replication within host macrophages is a crucial step for *Salmonella* to cause life-threatening systemic infection in the host (Bomjan, Zhang, & Zhou, 2019; Lathrop et al., 2015), while the crosstalk between *Salmonella* and macrophages at the metabolic interface is critical for intracellular *Salmonella* replication (Dandekar et al., 2014; Herrero-Fresno & Olsen, 2018; Lynch & Lesser, 2021; Rosenberg et al., 2021;

Thompson, Fulde, & Tedin, 2018). Emerging evidence suggests that several metabolites affect the replication of *Salmonella* in macrophages. The promotion of intracellular replication by metabolites is possibly achieved in three ways: i) metabolites are utilized by *Salmonella* as nutrients for intracellular growth (Bowden, Rowley, Hinton, & Thompson, 2009; Eisenreich, Dandekar, Heesemann, & Goebel, 2010; J. Wang et al., 2021); ii) *Salmonella* senses metabolites as environmental cues to activate the expression of virulence genes (Jiang et al., 2021; X. Wang et al., 2023); and iii) metabolites can regulate the immune responses of macrophages (Michelucci et al., 2013; Peace & O'Neill, 2022; Yang & Cong, 2021). In this study, we demonstrated that *Salmonella* promotes its replication inside macrophages by utilizing both host- and bacterial-derived β-alanine (Figure 6D). We showed that β-alanine promotes *Salmonella* intracellular replication and systemic infection partially by increasing the expression of zinc transporter genes and therefore the uptake of zinc by intracellular *Salmonella* (Figure 6D). Therefore, this work identified another metabolite that can influence the replication of *Salmonella* in macrophages and illustrated the mechanism by which β-alanine promotes intracellular *Salmonella* replication.

We observed that *Salmonella*-infected macrophages contained lower β-alanine levels than mock-infected macrophages, while β-alanine supplementation in the cell medium increased the replication of *Salmonella* in macrophages, revealing that *Salmonella* uptakes host-derived β-alanine to promote intracellular replication. In addition, a deficiency in the biosynthesis of β-alanine (*via* mutation of the

rate-limiting gene *panD*) reduced *Salmonella* replication in macrophages and systemic infection in mice, suggesting that *Salmonella* also utilizes bacterial-derived β-alanine to promote intracellular replication and pathogenicity. Therefore, we speculate that *Salmonella* relies on a large amount of β-alanine to efficiently replicate in macrophages, thereby highlighting the importance of β-alanine for *Salmonella* intracellular growth. Nevertheless, unlike the closely related species *E. coli*, which takes up β-alanine via the transporter protein CycA (Schneider et al., 2004), *Salmonella* does not use CycA to uptake β-alanine, implying the presence of other yet unknown β-alanine import mechanisms in *Salmonella*.

Several amino acids, including lysine, proline, arginine, aspartate and asparagine, have previously been reported to be associated with the pathogenicity of *Salmonella* (Popp et al., 2015; Steeb et al., 2013). These amino acids are involved in the synthesis of proteins in *Salmonella*. In contrast, β-alanine is not incorporated into proteins but can participate in the regulation of bacterial activity through the synthesis of pantothenate and CoA (Webb et al., 2004; White et al., 2001; Yuan et al., 2022). Accordingly, our transcriptome data showed that a deficiency in β-alanine biosynthesis affected the expression of *Salmonella* genes involved in a series of important metabolic pathways. Importantly, although β-alanine does not influence the gene expression of SPIs, it activates methionine metabolism; fatty acid β-oxidation; histidine biosynthesis; and the transport of zinc, galactose, potassium, and polyamine, which have been previously known to be associated with the virulence of *Salmonella* and other bacterial pathogens. Further analysis revealed that β-alanine promotes

Salmonella intracellular replication and systemic infection partially by promoting the uptake of zinc. As for the other virulence-related metabolic pathways activated by β-alanine, methionine metabolism, fatty acid β-oxidation, and histidine biosynthesis contribute to the virulence of *Streptococcus pneumoniae* (Basavanna et al., 2013), *Yersinia pestis* (Feldman et al., 2016), and *Acinetobacter baumannii* (Martínez-Guitián et al., 2019), respectively; the uptake of galactose and polyamine influences the pathogenicity of *Francisella tularensis* (Lauriano et al., 2004) and the avian pathogenic *Escherichia coli* (de Paiva et al., 2016), respectively; and the uptake of potassium is associated with the virulence of *Salmonella* Enteritidis in chickens (Liu et al., 2013). However, blocking these pathways did not influence the systemic *Salmonella* Typhimurium infection in mice, implying that different bacterial pathogens adopt different virulence strategies to establish infection. Determining other mechanism(s) by which β-alanine promotes the intracellular replication and systemic infection of *Salmonella* require further investigation.

We observed that β-alanine also activates the expression of the LysR-type transcriptional regulator LeuO, which is known to regulate the expression of a wide variety of *Salmonella* genes that impact the stress response and virulence (Dillon et al., 2012; Guadarrama et al., 2014; Hernández-Lucas et al., 2008). Typically, LeuO activates the synthesis of the quiescent porins OmpS1 and OmpS2, which are required for *Salmonella* virulence in mice (De la Cruz et al., 2007; Fernández-Mora, Puente, & Calva, 2004; Rodríguez-Morales et al., 2006). Consistent with the positive regulation of OmpS1 and OmpS2 by LeuO, lack of *leuO* in *Salmonella* also attenuated virulence

in a mouse model (Rodríguez-Morales et al., 2006). However, the attenuated phenotypes of the *leuO* mutant in mice were not evident after i.p. injection relative to oral infection, as a previous report showed that the competitive index for the *leuO* mutant indicated approximately 1,000-fold reduced colonization in mouse systemic tissues after oral infection but much less reduced colonization after i.p. injection (less than 10-fold) (Rodríguez-Morales et al., 2006). These results imply that LeuO might be predominantly associated with the invasion and intestinal infection of *Salmonella* but is weakly implicated in *Salmonella* intracellular replication and systemic infection. Therefore, it is not surprising that lack of *leuO* did not significantly affect *Salmonella* colonization in the systemic tissues of mice after i.p. injection, as revealed by our results.

Zinc is an essential micronutrient for all living organisms and is used as a cofactor for various enzymes and proteins (Bock, Müller, & Blankenfeldt, 2016; Ilari et al., 2016; Tan, Bramlett, & Lindahl, 2004). In bacteria, zinc-binding proteins account for approximately 5% of the bacterial proteome and play crucial roles in bacterial metabolism and virulence (Andreini, Banci, Bertini, & Rosato, 2006). Knockout of the zinc transporter ZnuABC reduces the virulence of *Salmonella*, *Campylobacter jejuni*, *Haemophilus ducreyi*, *Moraxella*, and urinary tract pathogenic *Escherichia coli* (UPEC) in the host (Ilari et al., 2016). Moreover, zinc is also utilized by *Salmonella* to subvert the antimicrobial host defense of macrophages by inhibiting NF-κB activation and impairing NF-κB-dependent bacterial clearance (Jennings, Esposito, Rittinger, & Thurston, 2018; Wu et al., 2017). In this work, we found that

β-alanine facilitates the pathogenicity of *Salmonella* by promoting the expression of zinc transporter genes. The results demonstrate a correlation between *Salmonella* β-alanine utilization and zinc uptake during intracellular infection and provide evidence that β-alanine can influence the macrophage immune response by acting on zinc uptake.

Overall, our findings suggest a model in which *Salmonella* exploits host- and bacterial-derived β-alanine to efficiently replicate in host macrophages and cause systemic disease. We propose that *Salmonella* requires a large amount of β-alanine during intracellular infection. The utilization of β-alanine promotes *Salmonella* uptake of the essential micronutrient zinc, which was previously shown to be required for the metabolic needs of intracellular *Salmonella* and to subvert the antimicrobial defense of macrophages by *Salmonella*. These observations provide new insight into *Salmonella* pathogenesis and the crosstalk between *Salmonella* and macrophages during intracellular infection. Considering that the *panD* gene is present in the genomes of all *Salmonella* strains and that mutation of *panD* markedly reduced *Salmonella* replication ability in macrophages, as well as virulence in the mouse model, this gene may be used as a potential target to control systemic *Salmonella* infection.

Methods

Ethics statement

Six-week-old female BALB/c mice were obtained from Beijing Vital River Laboratory Animal Technology (Beijing, China). Mice were housed in barrier facilities under specific pathogen-free conditions with a 12 h light/dark cycle at a temperature of 24 ± 2 °C and a relative humidity of 50 ± 5%. Mice were fed a standard mouse chow diet, and they consumed food and water *ad libitum* throughout the experiment. All animal experiments were conducted in accordance with the policies of the Institutional Animal Care Committee of Nankai University (Tianjin, China) and performed under protocol no. 2021-SYDWLL-000029.

Cell culture

The RAW264.7 mouse macrophage-like cell line (ATCC TIB-71) was obtained from the Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco #11879020) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco #10100147) at 37 °C with 5% CO_2. Cells were seeded in 24-well tissue culture plates at 1×10^5 cells per well 24 h before infection.

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. The *Salmonella enterica* serovar Typhimurium (STM) strain ATCC 14028s was used as the WT strain throughout this study and for the construction of the mutants. Mutant strains were generated using the λ Red recombination system with the plasmid pSIM17 (Jiang et al., 2017). To construct the complemented strain of Δ*panD*, the amplified DNA fragments of the *panD* ORF and its upstream promoter were digested

and inserted into the BamHI–EcoRI site of the low-copy-number plasmid pBR322. To generate the *panD-lux* reporter fusion, the amplification products of the *panD* promoter region were digested and cloned into the XhoI–BamHI site of the plasmid pMS402, which carries a promoter-less *lux*CDABE reporter gene cluster (Liang, Li, Dong, Surette, & Duan, 2008). The sequences of primers used for the construction of the strains are listed in Table S2. All the strains were verified by PCR amplification and sequencing.

Bacterial strains were conventionally grown overnight in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) or in N-minimal medium (10 μM $MgCl_2$, 110 μM KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 5 mM KCl, 38 mM glycerol, and 0.1% [w/v] casamino acids) supplemented with appropriate antibiotics at 37 °C with shaking at 180 rpm or on LB agar plates. All antibiotics were used at their standard concentrations (chloramphenicol, 25 μg/mL; kanamycin, 50 μg/mL; ampicillin, 100 μg/mL; gentamicin, 10 or 100 μg/mL) unless otherwise mentioned.

Growth curve

Bacterial strains were conventionally grown overnight in LB medium. The next day, they were subcultured (1:100) in new LB medium and RPMI-1640 medium or subcultured in N-minimal medium supplemented with glycerol or β-alanine as the sole carbon source at 37 °C with shaking at 180 rpm. To measure the growth of bacteria, 200 μL of the bacterial cultures were transferred to the microplate wells. The

absorbance (OD_{600}) of the bacteria was measured every half hour for 12 h with a Spark multimode microplate reader (Tecan).

Bioluminescent reporter assays

STM WT carrying the *panD-luxCDABE* fusion plasmid was conventionally grown overnight in LB medium, and the next day, the cells were subcultured (1:100) in new LB medium or N-minimal medium for 8 h. The luminescence of the cultured bacteria (200 μl) was measured with a Spark multimode microplate reader (Tecan). Moreover, the cultured bacteria (100 μl) were serially diluted and plated on LB agar plates to estimate bacterial CFUs. Bacterial CFUs were used to normalize luminescence values.

***Salmonella* infection of macrophages**

Bacterial strains were conventionally grown overnight in LB medium to the late stationary phase, and the next day, the bacteria were diluted to 2×10^6 CFUs/mL and opsonized in RPMI-1640 medium supplemented with 10% FBS for 15 min. The macrophage monolayers were infected with the opsonized bacteria culture (0.5 mL/well, multiplicity of infection (MOI) = 10) and centrifuged at 800×*g* for 5 min to synchronize infection. The infected cells were incubated for 30 min at 37 °C in 5% CO_2 and then washed three times with 1× PBS. Fresh RPMI-1640 medium containing 100 μg/mL gentamicin was added to the infected cells to kill extracellular bacteria. After 1 h, fresh RPMI-1640 medium containing 10 μg/mL gentamicin was added to the infected cells for the remainder of the experiment. To assess the intarcelluar growth of *Salmonella*, the infected cells were lysed with 1% Triton X-100 at 2 hpi and

20 hpi, and the abundance of the intracellular bacteria CFUs was estimated on LB agar plates. The relative fold replication of intracellular bacterial strains was denoted as the CFUs recovered at 20 hpi relative to those at 2 hpi. The relative fold change in replication was normalized to the number of RAW264.7 cells. When indicated, 1 mM β-alanine or 100 μM $ZnSO_4$ were added after 1 h of gentamicin treatment.

Targeted metabolomics analysis of amino acids in macrophages

RAW264.7 cells were mock-infected or infected with STM WT for 8 h. The cells were harvested and washed with precooled PBS solution to remove the medium. Cellular metabolites were extracted using ice-cold extraction solvent (40:40:20 vol/vol/vol acetonitrile:methanol:water, 0.1 M formic acid), incubated at -20 ℃ for 20 min, and then centrifuged for 10 min at 12,000×g and 4 ℃ to obtain the supernatant. Subsequently, the supernatant was transferred to an LC－MS vial and analyzed using ultrahigh-performance liquid chromatography (Acquity; Waters, Milford, MA, USA) coupled with mass spectrometry (Q Exactive Hybrid Quadrupole-Orbitrap; Thermo Fisher Scientific, Waltham, MA, USA). Metabolites were separated with a Luna NH_2 column (2 mm × 100 mm, 3 μm particle size; Phenomenex). Mobile phase A was 20 mM ammonium acetate (pH 9.0), and mobile phase B was acetonitrile containing 0.1% formic acid. The flow rate was 0.4 ml/min. Xcalibur 4.0 software (Thermo Fisher) was used for data acquisition and processing. Metabolite identification was achieved by high-resolution mass and retention time matching to authentic standards. The absolute quantification of amino acids was performed using the standard curve

method, and the values were normalized to the cell number. Four biological replicates of each sample were analyzed.

Immunofluorescence staining

RAW264.7 cells were infected with STM WT or Δ*panD* and the complemented strain c*panD* as described above. After 2 and 20 h of cultivation, the infected cells were fixed for 15 min in 4% paraformaldehyde, washed in PBS, and permeabilized with 0.1% Triton X-100 in PBS for 15 min. The fixed samples were blocked in 5% bovine serum albumin for 30 min, followed by staining with a FITC-conjugated anti-*Salmonella* antibody (1:100 dilution, Abcam #ab20320) for 1 h at room temperature in the dark. The nuclei were then stained with DAPI (Invitrogen #D21490) for 2 min. A confocal laser scanning microscope (Zeiss LSM800) and ZEN 2.3 software (blue edition) were used to acquire and analyze the cell images (Objective lense: 40×; The number of intracellular bacteria per infected cell was estimated in random fields by Fiji-ImageJ).

RNA isolation

RNA was extracted from *Salmonella* strains cultured in N-minimal medium or LB medium. To investigate the expression of the *Salmonella panD* gene inside macrophages, we obtained RNA from intracellular bacteria in RAW264.7 cells at 8 h postinfection and from bacteria in RPMI medium. RNA was extracted using an EASYspinPlus bacterial RNA rapid extraction kit (Aidlab #RN0802) according to the manufacturer's protocol. RNA quantity and purity were determined using a

NanoDrop 2000 spectrophotometer (NanoDrop Technologies). RNA samples were stored at −80 °C before use.

Quantitative real-time PCR (qRT–PCR)

According to the manufacturer's protocols, qRT–PCR was performed using 2× RealStar Power SYBR qPCR Mix (Genstar #A304) in a QuantStudio 5 Real Time PCR system (Applied Biosystems). cDNA was synthesized using a StarScript III RT Kit (Genstar #A232). Each sample was subjected to qRT–PCR in triplicate. The expression level of the 16S *rRNA* gene was used to normalize that of the target genes. We estimated the expression of each target gene using the $2^{-\Delta\Delta Ct}$ method.

RNA sequencing and analyses

The STM WT and Δ*panD* strains were conventionally grown overnight in LB medium, subcultured (1:100) in N-minimal medium for 8 h and then collected by centrifugation for RNA extraction. Sequencing libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer's instructions, and sequencing was conducted using the Illumina HiSeq 2000 platform at Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The sequencing data were deposited in the NCBI Sequence Read Archive under accession number (SRA, PRJNA1124637). The clean reads were mapped to the STM ATCC 14028 reference genome (CP001363 and CP001362) by using the short-sequence alignment software Bowtie 2. Gene expression was evaluated using the fragments per kilobase of transcript per million mapped reads (FPKM) method. DEGs in the *panD* mutant relative to the WT were determined using

the R statistical package software EdgeR. The thresholds for statistically significant differences were set to a fold change ≥ 2 or ≤ 0.5 and a false discovery rate (FDR) $\leqslant$ 0.05. P values were adjusted using the Benjamini–Hochberg procedure for controlling the FDR. Enrichment analysis of DEGs was conducted using GO and KEGG enrichment analyses.

Mouse infection

Salmonella strains were conventionally cultured overnight in LB medium, and the next day, they were subcultured (1:100) in new LB medium and grown at 37 °C with shaking at 200 rpm to stationary phase (OD_{600}~2). The bacteria were diluted to 5×10^4 CFUs/mL in 0.9% NaCl. Groups of BALB/c mice were infected i.p. with 0.1 mL of the NaCl suspension. For survival assays, we recorded and monitored the mortality and body weight of the infected mice daily for 10 days. To analyze the bacterial burden of the mouse liver and spleen, we weighed the infected mice first on day 3 postinfection and then harvested the liver and spleen. The liver and spleen of infected mice were homogenized in ice-cold PBS, serially diluted, and plated on LB plates containing the appropriate antibiotics to determine bacterial CFUs. H&E staining of the mouse liver was performed to investigate the histopathological alterations in the liver of infected mice. To evaluate the histopathological alterations in the mouse liver, we harvested the liver of the infected mice on day 5 postinfection. The mouse liver was washed with 0.9% NaCl, fixed with 10% neutral formalin for 48 h and subsequently processed for routine paraffin embedding. Paraffin-embedded tissues were sectioned at a thickness of 5 μm and then stained with hematoxylin

(Sangon Biotech #E607318) and eosin (Sangon Biotech #E607318) for histopathological examination. The stained sections were then examined by light microscopy (Leica DM2500 LED).

Flow cytometry

RAW264.7 cells were infected with *Salmonella* WT for 8 h, in the absence or presence of 1 mM β-alanine, which was added to the infected cells at 1 h post-infection. The infected cells were fixed for 15 min in 4% paraformaldehyde, washed in PBS, and permeabilized with 0.1% Triton X-100 in PBS for 15 min. The fixed samples were blocked in 5% bovine serum albumin for 30 min, followed by staining with an anti-CD86 antibody (Abcam, ab119857), an anti-CD163 antibody (Abcam, ab182422) for 30 min, and a goat anti-rat IgG H&L (Alexa Fluor® 488) (Abcam, ab150165), a donkey anti-rabbit IgG H&L (Alexa Fluor® 647) (Abcam, ab150075) for 30 min in the dark. The infected cells were analyzed using a BD FACSAria Flow Cytometer (BD Biosciences).

Statistical analysis

The data are presented as the mean ± SD. Statistical analyses were performed using GraphPad InStat software (version GraphPad Prism 9.5.1, San Diego, CA, USA) with two-sided Student's *t* tests, one-way ANOVA, two-way ANOVA, log-rank Mantel–Cox tests, or Mann–Whitney U tests according to the test requirements (as stated in the figure legends). A P value < 0.05 indicated a statistically significant difference. ns represents no statistical significance.

Data availability statement

The RNA-seq data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA1124637. All other data associated with this study are available in the main text and supplementary materials. Source data are provided in Supplementary Data 1.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC) Program (grant no. 32170110, 32270191), the Natural Science Foundation of Tianjin (grant no. 22JCYBJC01060), and the Fundamental Research Funds for the Central Universities, Nankai University (grant no. 63241588, 63243161).

Author contributions

LJ designed the research; SM, YS, XW, HG, RL, TY, CK, and JC performed the research; BY provided technical support and insights; SM and BY analyzed the data; and LJ and SM wrote the manuscript.

Disclosure statement

The authors report there are no competing interests to declare.

ORCID

Lingyan Jiang http://orcid.org/0000-0001-8580-606X

References

Ammendola, S., Pasquali, P., Pistoia, C., Petrucci, P., Petrarca, P., Rotilio, G., & Battistoni, A. (2007). High-affinity Zn2+ uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of *Salmonella enterica*. *Infect Immun, 75*(12), 5867-5876. doi:10.1128/iai.00559-07

Andreini, C., Banci, L., Bertini, I., & Rosato, A. (2006). Counting the zinc-proteins encoded in the human genome. *J Proteome Res, 5*(1), 196-201. doi:10.1021/pr050361j

Basavanna, S., Chimalapati, S., Maqbool, A., Rubbo, B., Yuste, J., Wilson, R. J., . . . Brown, J. S. (2013). The effects of methionine acquisition and synthesis on *Streptococcus pneumoniae* growth and virulence. *PLoS One, 8*(1), e49638. doi:10.1371/journal.pone.0049638

Battistoni, A., Ammendola, S., Chiancone, E., & Ilari, A. (2017). A novel antimicrobial approach based on the inhibition of zinc uptake in *Salmonella enterica*. *Future Med Chem, 9*(9), 899-910. doi:10.4155/fmc-2017-0042

Begley, T. P., Kinsland, C., & Strauss, E. (2001). The biosynthesis of coenzyme A in bacteria. *Vitam Horm, 61*, 157-171. doi:10.1016/s0083-6729(01)61005-7

Bellinger, P. M. (2014). β-Alanine supplementation for athletic performance: an update. *J Strength Cond Res, 28*(6), 1751-1770. doi:10.1519/jsc.0000000000000327

Bock, T., Müller, R., & Blankenfeldt, W. (2016). Crystal structure of AibC, a reductase involved in alternative de novo isovaleryl coenzyme A biosynthesis in *Myxococcus xanthus*. *Acta Crystallogr F Struct Biol Commun, 72*(Pt 8), 652-658. doi:10.1107/s2053230x16011146

Boldyrev, A. A., Aldini, G., & Derave, W. (2013). Physiology and pathophysiology of carnosine. *Physiol Rev, 93*(4), 1803-1845. doi:10.1152/physrev.00039.2012

Bomjan, R., Zhang, M., & Zhou, D. (2019). YshB promotes intracellular replication and is required for *salmonella* virulence. *J Bacteriol, 201*(17), e00314-19. doi:10.1128/jb.00314-19

Bowden, S. D., Rowley, G., Hinton, J. C., & Thompson, A. (2009). Glucose and glycolysis are required for the successful infection of macrophages and mice by *Salmonella enterica* serovar Typhimurium. *Infect Immun, 77*(7), 3117-3126. doi:10.1128/iai.00093-09

Castanheira, S., & García-Del Portillo, F. (2017). *Salmonella* populations inside host cells. *Front Cell Infect Microbiol, 7*, 432. doi:10.3389/fcimb.2017.00432

Dandekar, T., Fieselmann, A., Fischer, E., Popp, J., Hensel, M., & Noster, J. (2014). *Salmonella*-how a metabolic generalist adopts an intracellular lifestyle during infection. *Front Cell Infect Microbiol, 4*, 191. doi:10.3389/fcimb.2014.00191

Davaapil, H., Tsuchiya, Y., & Gout, I. (2014). Signalling functions of coenzyme A and its derivatives in mammalian cells. *Biochem Soc Trans, 42*(4), 1056-1062. doi:10.1042/bst20140146

De la Cruz, M. A., Fernández-Mora, M., Guadarrama, C., Flores-Valdez, M. A.,

Bustamante, V. H., Vázquez, A., & Calva, E. (2007). LeuO antagonizes H-NS and StpA-dependent repression in *Salmonella enterica* ompS1. *Mol Microbiol, 66*(3), 727-743. doi:10.1111/j.1365-2958.2007.05958.x

de Paiva, J. B., da Silva, L. P., Casas, M. R., Conceição, R. A., Nakazato, G., de Pace, F., . . . da Silveira, W. D. (2016). *In vivo* influence of in vitro up-regulated genes in the virulence of an APEC strain associated with swollen head syndrome. *Avian Pathol, 45*(1), 94-105. doi:10.1080/03079457.2015.1125995

Dillon, S. C., Espinosa, E., Hokamp, K., Ussery, D. W., Casadesús, J., & Dorman, C. J. (2012). LeuO is a global regulator of gene expression in *Salmonella enterica* serovar Typhimurium. *Mol Microbiol, 85*(6), 1072-1089. doi:10.1111/j.1365-2958.2012.08162.x

Eisenreich, W., Dandekar, T., Heesemann, J., & Goebel, W. (2010). Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. *Nat Rev Microbiol, 8*(6), 401-412. doi:10.1038/nrmicro2351

Fàbrega, A., & Vila, J. (2013). *Salmonella enterica* serovar Typhimurium skills to succeed in the host: virulence and regulation. *Clin Microbiol Rev, 26*(2), 308-341. doi:10.1128/cmr.00066-12

Feldman, M., Harbeck, M., Keller, M., Spyrou, M. A., Rott, A., Trautmann, B., . . . Krause, J. (2016). A High-Coverage *Yersinia pestis* Genome from a Sixth-Century Justinianic Plague Victim. *Mol Biol Evol, 33*(11), 2911-2923. doi:10.1093/molbev/msw170

Fernández-Mora, M., Puente, J. L., & Calva, E. (2004). OmpR and LeuO positively

regulate the *Salmonella enterica* serovar Typhi ompS2 porin gene. *J Bacteriol, 186*(10), 2909-2920. doi:10.1128/jb.186.10.2909-2920.2004

Fields, P. I., Swanson, R. V., Haidaris, C. G., & Heffron, F. (1986). Mutants of *Salmonella* Typhimurium that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci U S A, 83*(14), 5189-5193. doi:10.1073/pnas.83.14.5189

Figueira, R., & Holden, D. W. (2012). Functions of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology (Reading), 158*(Pt 5), 1147-1161. doi:10.1099/mic.0.058115-0

Gout, I. (2019). Coenzyme A: a protective thiol in bacterial antioxidant defence. *Biochem Soc Trans, 47*(1), 469-476. doi:10.1042/bst20180415

Guadarrama, C., Villaseñor, T., & Calva, E. (2014). The subtleties and contrasts of the LeuO regulator in *Salmonella* Typhi: implications in the immune response. *Front Immunol, 5*, 581. doi:10.3389/fimmu.2014.00581

Hernández-Lucas, I., Gallego-Hernández, A. L., Encarnación, S., Fernández-Mora, M., Martínez-Batallar, A. G., Salgado, H., . . . Calva, E. (2008). The LysR-type transcriptional regulator LeuO controls expression of several genes in *Salmonella enterica* serovar Typhi. *J Bacteriol, 190*(5), 1658-1670. doi:10.1128/jb.01649-07

Herrero-Fresno, A., & Olsen, J. E. (2018). *Salmonella* Typhimurium metabolism affects virulence in the host - A mini-review. *Food Microbiol, 71*, 98-110. doi:10.1016/j.fm.2017.04.016

Hoffman, J. R., Varanoske, A., & Stout, J. R. (2018). Effects of β-Alanine supplementation on carnosine elevation and physiological performance. *Adv Food Nutr Res, 84*, 183-206. doi:10.1016/bs.afnr.2017.12.003

Huerta Ojeda, Á., Tapia Cerda, C., Poblete Salvatierra, M. F., Barahona-Fuentes, G., & Jorquera Aguilera, C. (2020). Effects of beta-Alanine supplementation on physical performance in aerobic-anaerobic transition zones: a systematic review and meta-analysis. *Nutrients, 12*(9), 2490. doi:10.3390/nu12092490

Ilari, A., Pescatori, L., Di Santo, R., Battistoni, A., Ammendola, S., Falconi, M., . . . Chiancone, E. (2016). *Salmonella enterica* serovar Typhimurium growth is inhibited by the concomitant binding of Zn(II) and a pyrrolyl-hydroxamate to ZnuA, the soluble component of the ZnuABC transporter. *Biochim Biophys Acta, 1860*(3), 534-541. doi:10.1016/j.bbagen.2015.12.006

Jennings, E., Esposito, D., Rittinger, K., & Thurston, T. L. M. (2018). Structure-function analyses of the bacterial zinc metalloprotease effector protein GtgA uncover key residues required for deactivating NF-κB. *J Biol Chem, 293*(39), 15316-15329. doi:10.1074/jbc.RA118.004255

Jiang, L., Feng, L., Yang, B., Zhang, W., Wang, P., Jiang, X., & Wang, L. (2017). Signal transduction pathway mediated by the novel regulator LoiA for low oxygen tension induced Salmonella Typhimurium invasion. *PLoS Pathog, 13*(6), e1006429. doi:10.1371/journal.ppat.1006429

Jiang, L., Wang, P., Song, X., Zhang, H., Ma, S., Wang, J., . . . Wang, L. (2021). *Salmonella* Typhimurium reprograms macrophage metabolism via T3SS

effector SopE2 to promote intracellular replication and virulence. *Nat Commun, 12*(1), 879. doi:10.1038/s41467-021-21186-4

Kim, S., Lee, K. S., Pak, G. D., Excler, J. L., Sahastrabuddhe, S., Marks, F., . . . Mogasale, V. (2019). Spatial and temporal patterns of typhoid and paratyphoid fever outbreaks: a worldwide review, 1990-2018. *Clin Infect Dis, 69*(Suppl 6), S499-s509. doi:10.1093/cid/ciz705

LaRock, D. L., Chaudhary, A., & Miller, S. I. (2015). Salmonellae interactions with host processes. *Nat Rev Microbiol, 13*(4), 191-205. doi:10.1038/nrmicro3420

Lathrop, S. K., Binder, K. A., Starr, T., Cooper, K. G., Chong, A., Carmody, A. B., & Steele-Mortimer, O. (2015). Replication of *Salmonella enterica* serovar Typhimurium in human monocyte-derived macrophages. *Infect Immun, 83*(7), 2661-2671. doi:10.1128/iai.00033-15

Lauriano, C. M., Barker, J. R., Yoon, S. S., Nano, F. E., Arulanandam, B. P., Hassett, D. J., & Klose, K. E. (2004). MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. *Proc Natl Acad Sci U S A, 101*(12), 4246-4249. doi:10.1073/pnas.0307690101

Leung, K. Y., & Finlay, B. B. (1991). Intracellular replication is essential for the virulence of *Salmonella* Typhimurium. *Proc Natl Acad Sci U S A, 88*(24), 11470-11474. doi:10.1073/pnas.88.24.11470

Li, W., Ren, Q., Ni, T., Zhao, Y., Sang, Z., Luo, R., . . . Li, S. (2023). Strategies adopted by *Salmonella* to survive in host: a review. *Arch Microbiol, 205*(12),

362. doi:10.1007/s00203-023-03702-w

Liang, H., Li, L., Dong, Z., Surette, M. G., & Duan, K. (2008). The YebC family protein PA0964 negatively regulates the Pseudomonas aeruginosa quinolone signal system and pyocyanin production. *J Bacteriol, 190*(18), 6217-6227. doi:10.1128/jb.00428-08

Liu, Y., Ho, K. K., Su, J., Gong, H., Chang, A. C., & Lu, S. (2013). Potassium transport of *Salmonella* is important for type III secretion and pathogenesis. *Microbiology (Reading), 159*(Pt 8), 1705-1719. doi:10.1099/mic.0.068700-0

Lynch, J. P., & Lesser, C. F. (2021). A host metabolite promotes *Salmonella* survival. *Science, 371*(6527), 344-345. doi:10.1126/science.abf8414

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., . . . Hoekstra, R. M. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin Infect Dis, 50*(6), 882-889. doi:10.1086/650733

Martínez-Guitián, M., Vázquez-Ucha, J. C., Álvarez-Fraga, L., Conde-Pérez, K., Lasarte-Monterrubio, C., Vallejo, J. A., . . . Beceiro, A. (2019). Involvement of HisF in the persistence of *Acinetobacter baumannii* during a pneumonia infection. *Front Cell Infect Microbiol, 9*, 310. doi:10.3389/fcimb.2019.00310

Michelucci, A., Cordes, T., Ghelfi, J., Pailot, A., Reiling, N., Goldmann, O., . . . Hiller, K. (2013). Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. *Proc Natl Acad Sci U S A, 110*(19), 7820-7825. doi:10.1073/pnas.1218599110

Ohl, M. E., & Miller, S. I. (2001). *Salmonella*: a model for bacterial pathogenesis.

Annu Rev Med, 52, 259-274. doi:10.1146/annurev.med.52.1.259

Peace, C. G., & O'Neill, L. A. (2022). The role of itaconate in host defense and inflammation. *J Clin Invest, 132*(2), e148548. doi:10.1172/jci148548

Pillay, T. D., Hettiarachchi, S. U., Gan, J., Diaz-Del-Olmo, I., Yu, X. J., Muench, J. H., . . . Pearson, J. S. (2023). Speaking the host language: how *Salmonella* effector proteins manipulate the host. *Microbiology (Reading), 169*(6), 001342. doi:10.1099/mic.0.001342

Popp, J., Noster, J., Busch, K., Kehl, A., Zur Hellen, G., & Hensel, M. (2015). Role of host cell-derived amino acids in nutrition of intracellular *Salmonella enterica*. *Infect Immun, 83*(12), 4466-4475. doi:10.1128/iai.00624-15

Röder, J., Felgner, P., & Hensel, M. (2021). Comprehensive single cell analyses of the nutritional environment of intracellular *Salmonella enterica*. *Front Cell Infect Microbiol, 11*, 624650. doi:10.3389/fcimb.2021.624650

Rodríguez-Morales, O., Fernández-Mora, M., Hernández-Lucas, I., Vázquez, A., Puente, J. L., & Calva, E. (2006). *Salmonella enterica* serovar Typhimurium ompS1 and ompS2 mutants are attenuated for virulence in mice. *Infect Immun, 74*(2), 1398-1402. doi:10.1128/iai.74.2.1398-1402.2006

Rosenberg, G., Riquelme, S., Prince, A., & Avraham, R. (2022). Immunometabolic crosstalk during bacterial infection. *Nat Microbiol, 7*(4), 497-507. doi:10.1038/s41564-022-01080-5

Rosenberg, G., Yehezkel, D., Hoffman, D., Mattioli, C. C., Fremder, M., Ben-Arosh, H., . . . Avraham, R. (2021). Host succinate is an activation signal for

Salmonella virulence during intracellular infection. *Science, 371*(6527), 400-405. doi:10.1126/science.aba8026

Schmitzberger, F., Smith, A. G., Abell, C., & Blundell, T. L. (2003). Comparative analysis of the *Escherichia coli* ketopantoate hydroxymethyltransferase crystal structure confirms that it is a member of the (betaalpha)8 phosphoenolpyruvate/pyruvate superfamily. *J Bacteriol, 185*(14), 4163-4171. doi:10.1128/jb.185.14.4163-4171.2003

Schneider, F., Krämer, R., & Burkovski, A. (2004). Identification and characterization of the main beta-alanine uptake system in *Escherichia coli*. *Appl Microbiol Biotechnol, 65*(5), 576-582. doi:10.1007/s00253-004-1636-0

Sibon, O. C., & Strauss, E. (2016). Coenzyme A: to make it or uptake it? *Nat Rev Mol Cell Biol, 17*(10), 605-606. doi:10.1038/nrm.2016.110

Steeb, B., Claudi, B., Burton, N. A., Tienz, P., Schmidt, A., Farhan, H., . . . Bumann, D. (2013). Parallel exploitation of diverse host nutrients enhances *Salmonella* virulence. *PLoS Pathog, 9*(4), e1003301. doi:10.1371/journal.ppat.1003301

Tan, X., Bramlett, M. R., & Lindahl, P. A. (2004). Effect of Zn on acetyl coenzyme a synthase: evidence for a conformational change in the alpha subunit during catalysis. *J Am Chem Soc, 126*(19), 5954-5955. doi:10.1021/ja039600z

Theodoulou, F. L., Sibon, O. C., Jackowski, S., & Gout, I. (2014). Coenzyme A and its derivatives: renaissance of a textbook classic. *Biochem Soc Trans, 42*(4), 1025-1032. doi:10.1042/bst20140176

Thompson, A., Fulde, M., & Tedin, K. (2018). The metabolic pathways utilized by

Salmonella Typhimurium during infection of host cells. *Environ Microbiol Rep, 10*(2), 140-154. doi:10.1111/1758-2229.12628

Tuli, A., & Sharma, M. (2019). How to do business with lysosomes: *Salmonella* leads the way. *Curr Opin Microbiol, 47*, 1-7. doi:10.1016/j.mib.2018.10.003

Wang, J., Ma, S., Li, W., Wang, X., Huang, D., Jiang, L., & Feng, L. (2021). *Salmonella enterica* serovar Typhi induces host metabolic reprogramming to increase glucose availability for intracellular replication. *Int J Mol Sci, 22*(18), 10003. doi:10.3390/ijms221810003

Wang, X., Yang, B., Ma, S., Yan, X., Ma, S., Sun, H., . . . Jiang, L. (2023). Lactate promotes *Salmonella* intracellular replication and systemic infection via driving macrophage M2 polarization. *Microbiol Spectr, 11*(6), e0225323. doi:10.1128/spectrum.02253-23

Webb, M. E., Smith, A. G., & Abell, C. (2004). Biosynthesis of pantothenate. *Nat Prod Rep, 21*(6), 695-721. doi:10.1039/b316419p

White, W. H., Gunyuzlu, P. L., & Toyn, J. H. (2001). *Saccharomyces cerevisiae* is capable of de *novo* pantothenic acid biosynthesis involving a novel pathway of beta-alanine production from spermine. *J Biol Chem, 276*(14), 10794-10800. doi:10.1074/jbc.M009804200

Wu, A., Tymoszuk, P., Haschka, D., Heeke, S., Dichtl, S., Petzer, V., . . . Weiss, G. (2017). *Salmonella* utilizes zinc to subvert antimicrobial host defense of macrophages via modulation of NF-κB signaling. *Infect Immun, 85*(12), e00418-17. doi:10.1128/iai.00418-17

Yang, W., & Cong, Y. (2021). Gut microbiota-derived metabolites in the regulation of host immune responses and immune-related inflammatory diseases. *Cell Mol Immunol, 18*(4), 866-877. doi:10.1038/s41423-021-00661-4

Yuan, S. F., Nair, P. H., Borbon, D., Coleman, S. M., Fan, P. H., Lin, W. L., & Alper, H. S. (2022). Metabolic engineering of E. coli for β-alanine production using a multi-biosensor enabled approach. *Metab Eng, 74*, 24-35. doi:10.1016/j.ymben.2022.08.012

Figure legends

Figure 1. Host-derived β-alanine promotes *Salmonella* replication inside macrophages. (A) Schematic workflow for targeted metabolomics investigation of mock- and *Salmonella*-infected (STM) mouse RAW264.7 macrophages. Picture materials were used from bioicons (https://bioicons.com/). (B) Principal component analysis (PCA) score plots of metabolic profiles in the mock- and *Salmonella*-infected (STM) groups (n = 4 biologically independent samples). (C) Relative copiousness of amino acids that were differentially abundant in the *Salmonella*-infected groups versus the mock groups. (D) The concentrations of β-alanine in the mock- and *Salmonella*-infected groups. (E) Relative fold replication of *Salmonella* WT in RAW264.7 cells in the presence or absence of 1 mM β-alanine. (F) qRT–PCR analysis of the expression of pro- (*Il1b*, *Tnf*) and anti-inflammatory genes (*Il10*, *Il4ra*) of RAW264.7 cells after infection with *Salmonella* WT for 8 h, in the presence or absence of 1 mM β-alanine. (G) Flow cytometry analysis of percentage of pro-inflammatory M1 macrophages (CD86+) and anti-inflammatory M2 macrophages (CD163+) . RAW264.7 cell were infected *Salmonella* WT for 8 h, in the presence or absence of 1 mM β-alanine, and then the infected cells were collected for flow cytometry analysis. Representative dot plot and quantification of M1 (CD86+) or M2 (CD163+) macrophages are shown in the left and right panel, respectively. The data are presented as the mean ± SD, n = 3 independent experiments (E-G). Statistical significance was assessed using two-sided Student's *t*-test (D, E) and two-way ANOVA (F, G)

Figure 2. De novo β-alanine synthesis is critical for *Salmonella* replication inside macrophages. (A) Scheme of β-alanine and the downstream CoA biosynthesis pathway in *Salmonella*. (B) qRT–PCR analysis of the expression of the *Salmonella panD* gene in RAW264.7 cells (8 h postinfection) and RPMI-1640 medium. (C) qRT–PCR analysis of the expression of the *Salmonella panD* gene in N-minimal medium and LB medium. (D) Expression of the *panD*-lux transcriptional fusion in N-minimal medium and LB medium. Luminescence values were normalized to 10^5 bacterial CFUs. (E) Relative fold replication of *Salmonella* WT, the *panD* mutant (Δ*panD*) and the complemented strain (c*panD*) in RAW264.7 cells. (F) Number of intracellular *Salmonella* WT, Δ*panD*, and c*panD* strains per RAW264.7 cell at 2 and 20 h postinfection. The number of intracellular bacteria per infected cell was estimated in random fields, n = 80 cells per group from 3 independent experiments. (G) Representative immunofluorescence images of *Salmonella* WT, Δ*panD*, and c*panD* in RAW264.7 cells at 20 h postinfection (green, *Salmonella*; blue, nuclei; scale bars, 50 μm). Images are representative of three independent experiments. (H) Replication of *Salmonella* WT and Δ*panD* in RAW264.7 cells in the presence or absence of 1 mM β-alanine. The data are presented as the mean ± SD, n = 3 (B–D) or n =4 (E, H) independent experiments. Statistical significance was assessed using two-sided Student's *t*-test (B-D) or one-way ANOVA (E, F, H). ns, not Significant.

Figure 3. De novo β-alanine synthesis is critical for systemic *Salmonella* infection in mice. (A) Schematic illustration of the mouse infection assays. Picture materials

were used from bioicons (https://bioicons.com/). (B, C) Survival curves (B) and body weight dynamics (C) of mice infected i.p. with *Salmonella* WT, Δ*panD*, or c*panD*. n = 10 mice per group. (D) Liver and spleen bacterial burdens and body weights of mice infected with *Salmonella* WT, Δ*panD*, or c*panD* on day 3 postinfection. n = 7 mice per group. (E) Representative H&E-stained liver sections from mice that were left uninfected or infected with *Salmonella* WT, Δ*panD*, or c*panD* on day 5 postinfection. Arrows indicate severe inflammatory cell infiltration in the mouse liver. Images are representative of three independent experiments. The data are presented as the mean ± SD (B-D). Statistical significance was assessed using the log-rank Mantel–Cox test (B), two-sided Student's *t*-test (C), or one-way ANOVA (D).

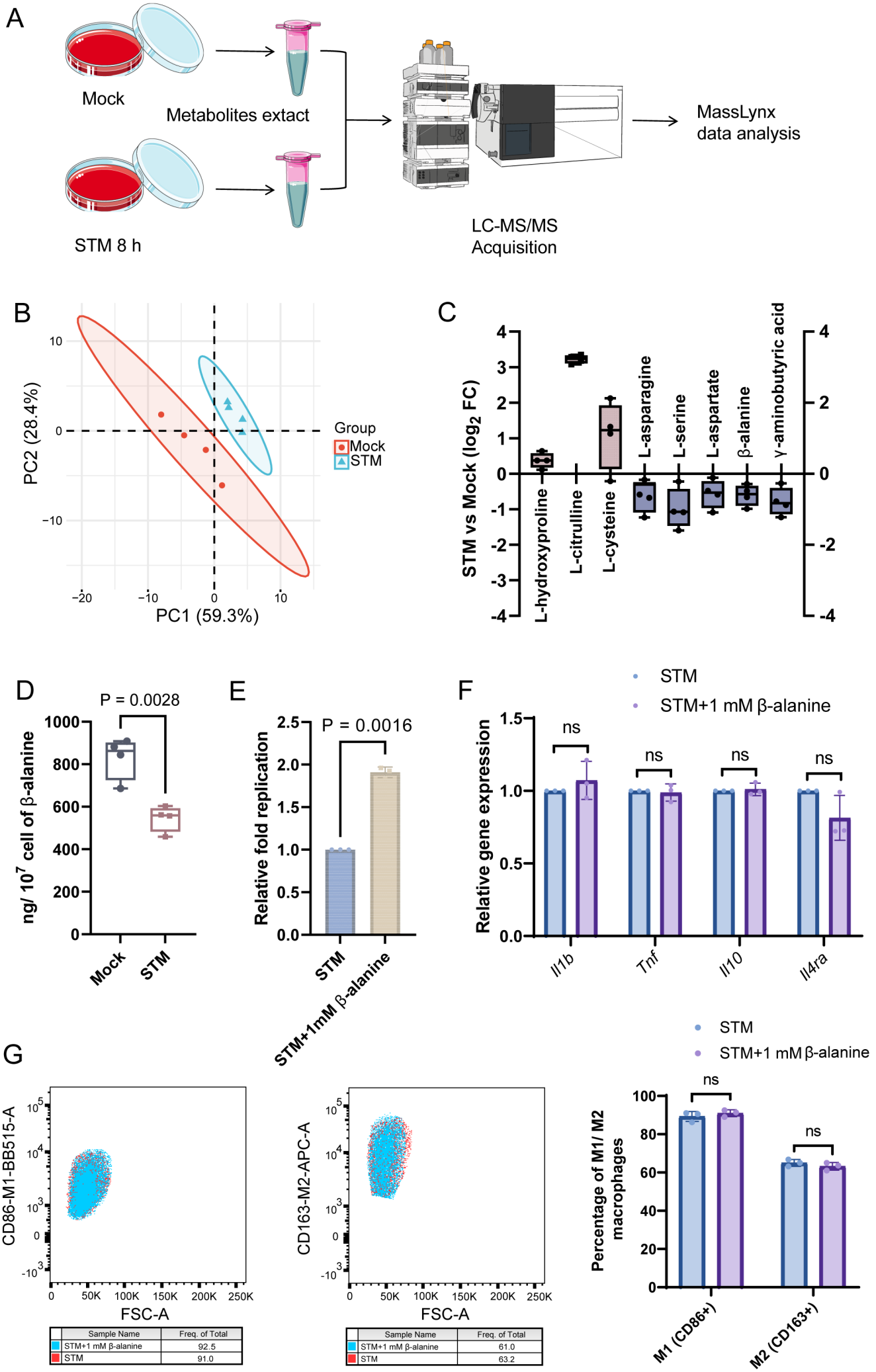
Figure 4. β-Alanine is involved in the regulation of several metabolic pathways in *Salmonella.* (A) Principal component analysis (PCA) score plots of transcriptomic profiles of *Salmonella* WT and Δ*panD* (n = 3 biologically independent samples). (B) Volcano plot of the differentially expressed genes (DEGs) in *Salmonella* WT versus Δ*panD*. The upper right section (red dots) indicates the upregulated DEGs, and the upper left section (green dots) indicates the downregulated DEGs. (C) Gene Ontology (GO) enrichment analysis of DEGs. Bubble chart showing the top 20 enriched GO terms. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs. (E) Expression of the downregulated pathways (activated by PanD) is shown in the Z score-transformed heatmap, with red representing higher abundance and blue representing lower abundance. (F) qRT–PCR analysis of the mRNA levels of

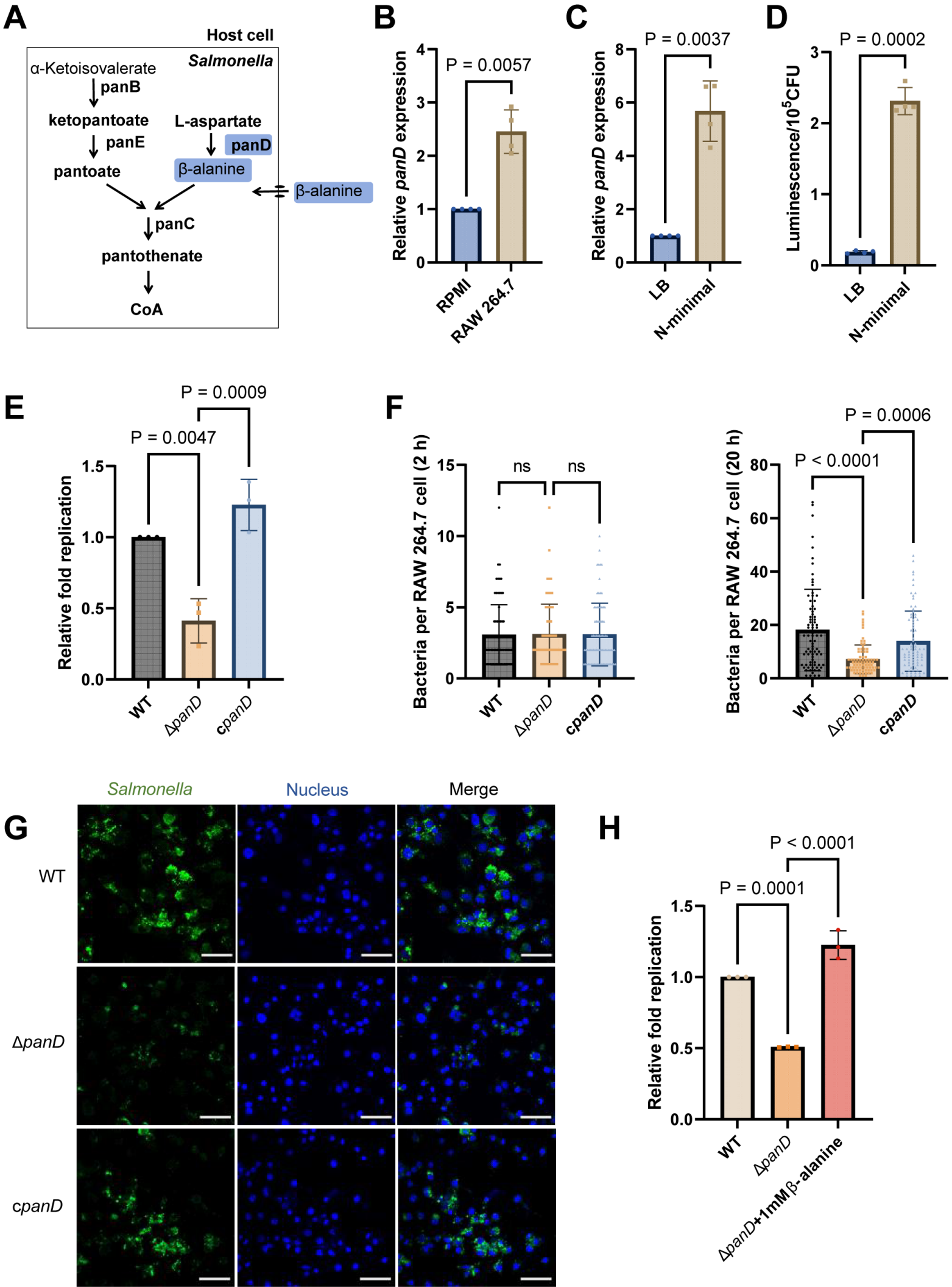
16 selected downregulated DEGs in *Salmonella* WT, Δ*panD*, and c*panD*. The data are presented as the mean ± SD, n = 3 independent experiments. Statistical significance was assessed using two-way ANOVA.

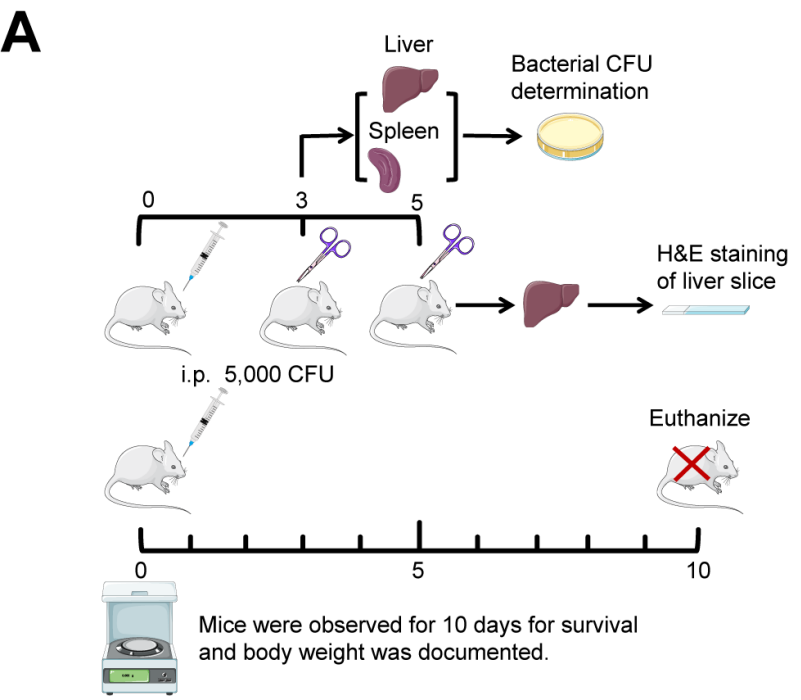
Figure 5. β-Alanine promotes *Salmonella* virulence *in vivo* by increasing the expression of zinc transporter genes. (A, B, C) Liver (A) and spleen (B) bacterial burdens and body weight (C) of mice infected with *Salmonella* WT, Δ*fadAB*, Δ*metR*, Δ*hisABCDFGHL*, Δ*kdpABC*, Δ*mglABC*, Δ*potFGHI*, or Δ*leuO* on day 3 postinfection. n = 5 mice per group. (D) Liver and spleen bacterial burdens and body weights of mice infected with *Salmonella* WT, Δ*panD*, Δ*znuA* or Δ*panD*Δ*znuA* on day 3 postinfection. n = 5 mice per group. The data are presented as the mean ± SD (A–D). Statistical significance was assessed using one-way ANOVA (A–D). ns, not Significant.

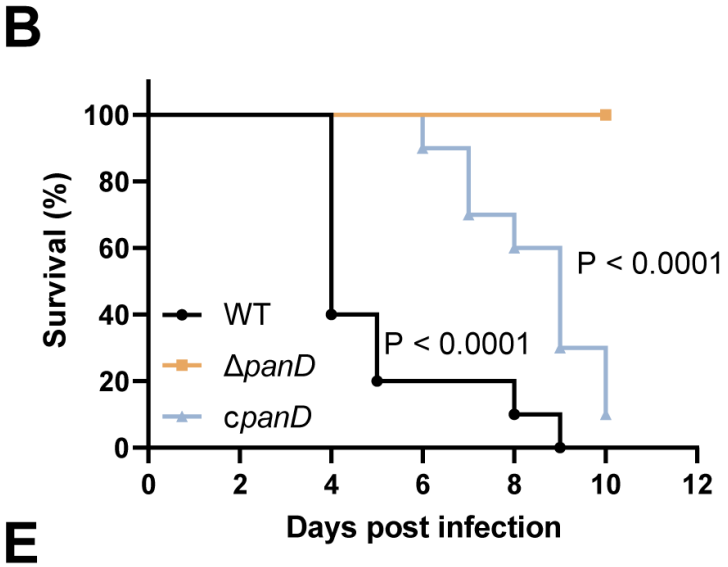
Figure 6. β-Alanine promotes *Salmonella* replication within macrophages by increasing the expression of zinc transporter genes. (A) Replication of *Salmonella* WT, Δ*panD*, Δ*znuA* and Δ*panD*Δ*znuA* in RAW264.7 cells. (B) Replication of *Salmonella* WT and Δ*panD* in RAW264.7 cells in the presence or absence of 100 μM $ZnSO_4$. (C) Replication of *Salmonella* WT and Δ*znuA* in RAW264.7 cells in the presence or absence of 1 mM β-alanine. The data are presented as the mean ± SD, n = 3 independent experiments (A–C). Statistical significance was assessed using one-way ANOVA (A–C). ns, not Significant. (D) Schematic model of

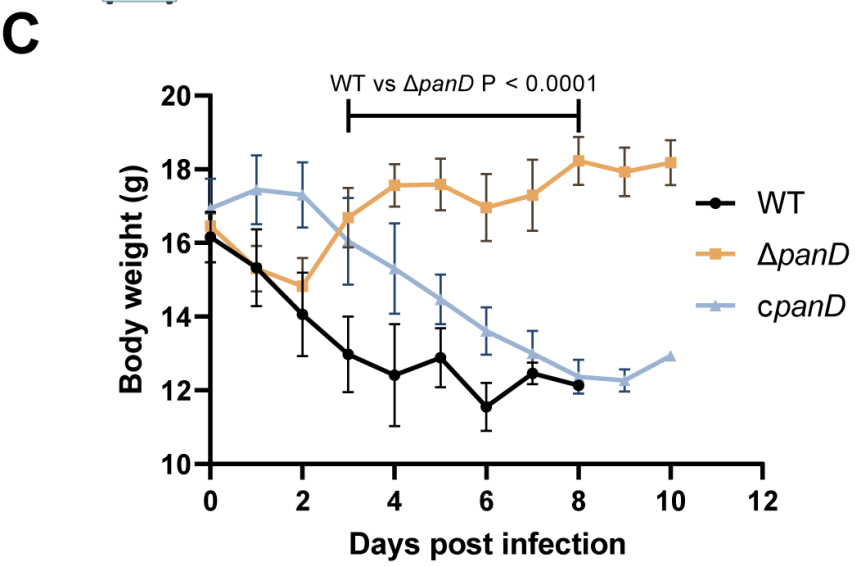
β-alanine-mediated *Salmonella* replication inside macrophages. In macrophages, *Salmonella* acquires β-alanine both via the uptake of β-alanine from host macrophages and the de novo synthesis of β-alanine. β-alanine promotes the expression of zinc transporter genes *ZnuABC*, which facilitate the uptake of zinc by intracellular *Salmonella*, therefore promote *Salmonella* replication in macrophages and subsequent systemic infection.

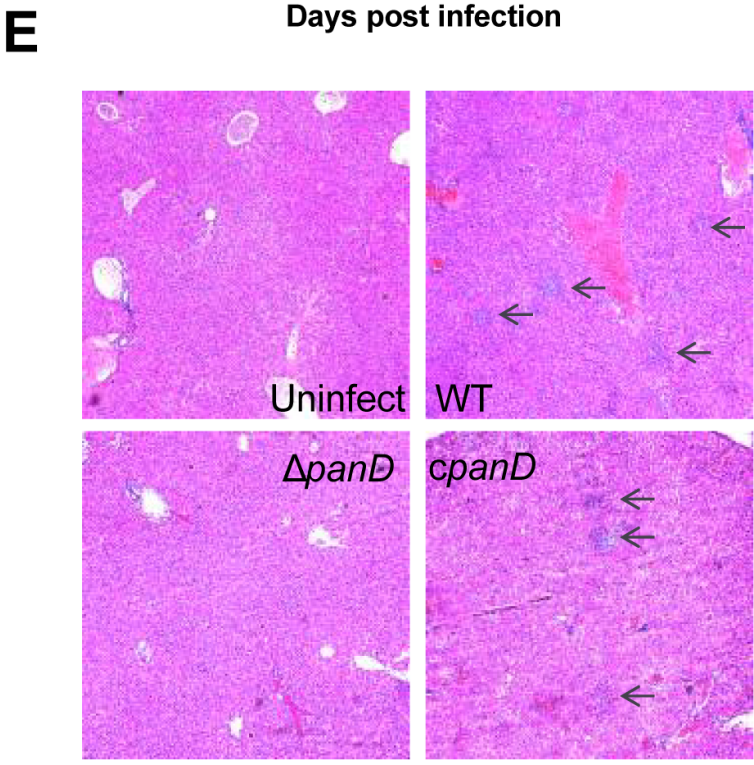

A
Mock
Metabolites extact
STM 8 h
LC-MS/MS
Acquisition
MassLynx
data analysis
B
PC2 (28.4%)
PC1 (59.3%)
Group
Mock
STM
C
STM vs Mock (log2 FC)
L-hydroxyproline
L-citrulline
L-cysteine
L-asparagine
L-serine
L-aspartate
β-alanine
γ-aminobutyric acid
D
P = 0.0028
ng/ 10^7 cell of β-alanine
Mock
STM
E
P = 0.0016
Relative fold replication
STM
STM+1mM β-alanine
F
STM
STM+1 mM β-alanine
Relative gene expression
ns
Il1b
Tnf
Il10
Il4ra
G
CD86-M1-BB515-A
CD163-M2-APC-A
FSC-A
Sample Name
Freq. of Total
STM+1 mM β-alanine
92.5
STM
91.0
STM+1 mM β-alanine
61.0
STM
63.2
Percentage of M1/ M2 macrophages
M1 (CD86+)
M2 (CD163+)

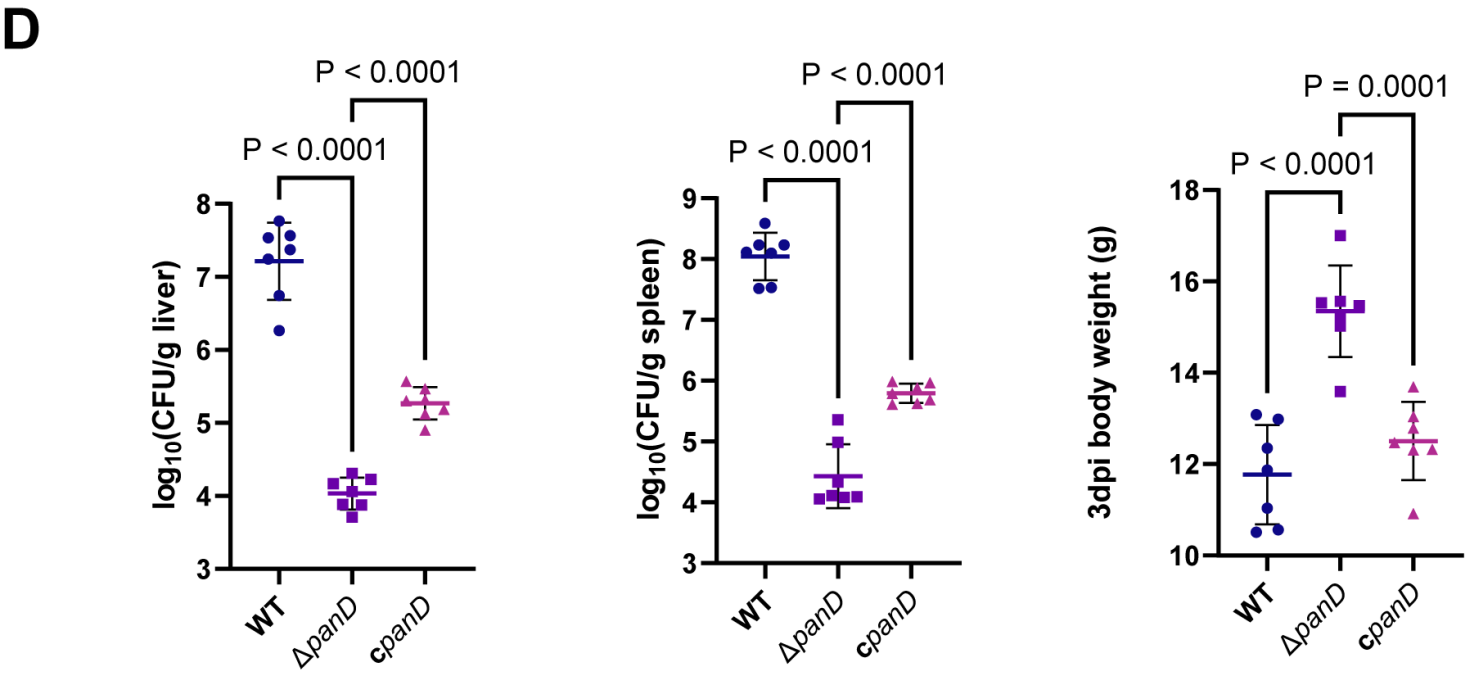

A
Host cell
Salmonella
α-Ketoisovalerate
panB
ketopantoate
panE
pantoate
L-aspartate
panD
β-alanine
β-alanine
panC
pantothenate
CoA
B
P = 0.0057
Relative panD expression
RPMI
RAW 264.7
C
P = 0.0037
Relative panD expression
LB
N-minimal
D
P = 0.0002
Luminescence/10^5CFU
LB
N-minimal
E
P = 0.0009
P = 0.0047
Relative fold replication
WT
ΔpanD
cpanD
F
ns
ns
Bacteria per RAW 264.7 cell (2 h)
WT
ΔpanD
cpanD
P = 0.0006
P < 0.0001
Bacteria per RAW 264.7 cell (20 h)
WT
ΔpanD
cpanD
G
Salmonella
Nucleus
Merge
WT
ΔpanD
cpanD
H
P < 0.0001
P = 0.0001
Relative fold replication
WT
ΔpanD
ΔpanD+1mM β-alanine

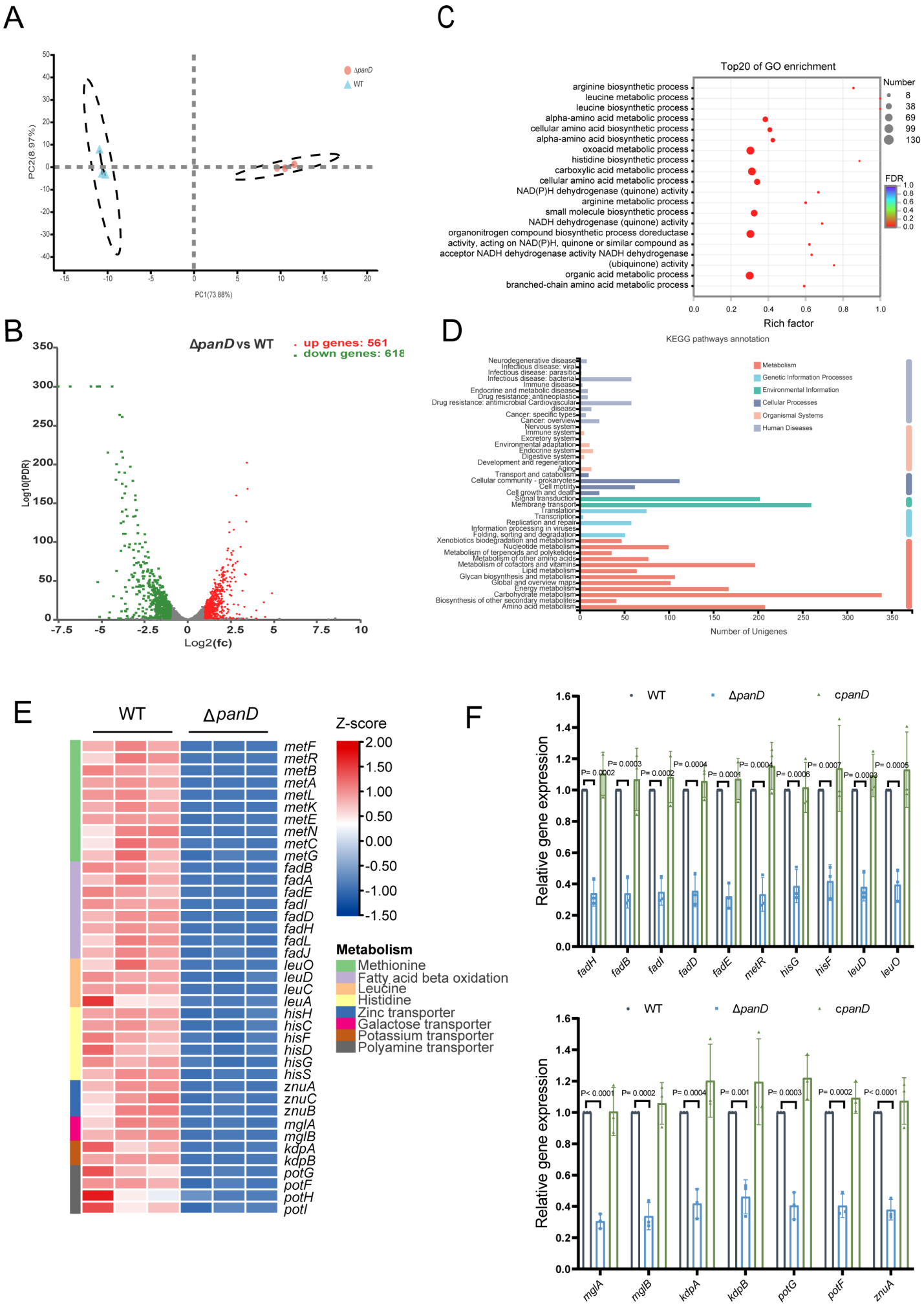

A
Liver
Spleen
Bacterial CFU determination
0
3
5
H&E staining of liver slice
i.p. 5,000 CFU
Euthanize
0
5
10
Mice were observed for 10 days for survival and body weight was documented.

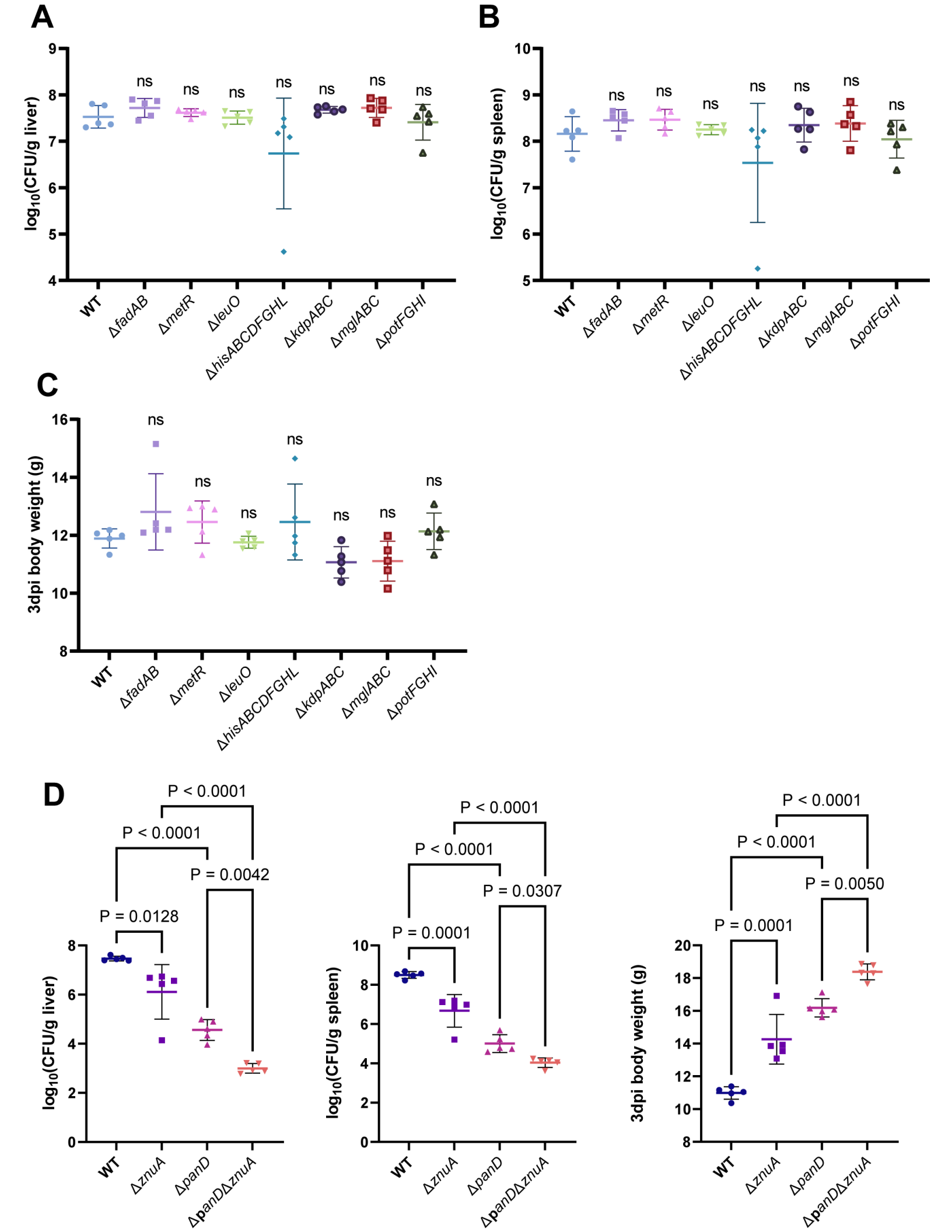

B
Survival (%)
100
80
60
40
20
0
WT
ΔpanD
cpanD
P < 0.0001
P < 0.0001
0
2
4
6
8
10
12
Days post infection

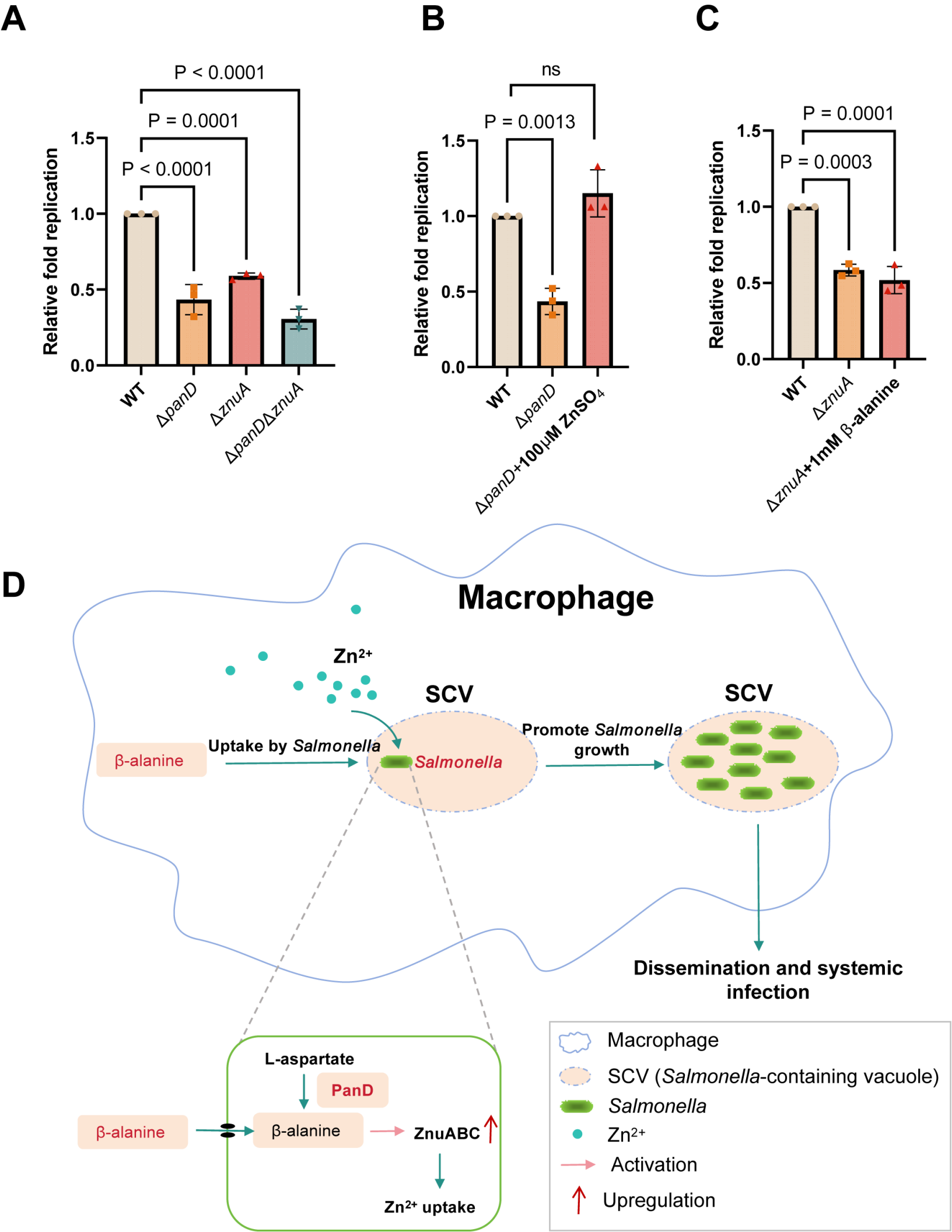

C
WT vs ΔpanD P < 0.0001
Body weight (g)
20
18
16
14
12
10
WT
ΔpanD
cpanD
0
2
4
6
8
10
12
Days post infection


E
Uninfect
WT
ΔpanD
cpanD


D
P < 0.0001
P < 0.0001
log10(CFU/g liver)
8
7
6
5
4
3
WT
ΔpanD
cpanD
P < 0.0001
P < 0.0001
log10(CFU/g spleen)
9
8
7
6
5
4
3
WT
ΔpanD
cpanD
P = 0.0001
P < 0.0001
3dpi body weight (g)
18
16
14
12
10
WT
ΔpanD
cpanD


A
ΔpanD
WT
PC2(8.97%)
PC1(73.88%)
B
ΔpanD vs WT
up genes: 561
down genes: 618
Log10(PDR)
Log2(fc)
C
Top20 of GO enrichment
arginine biosynthetic process
leucine metabolic process
leucine biosynthetic process
alpha-amino acid metabolic process
cellular amino acid biosynthetic process
alpha-amino acid biosynthetic process
oxoacid metabolic process
histidine biosynthetic process
carboxylic acid metabolic process
cellular amino acid metabolic process
NAD(P)H dehydrogenase (quinone) activity
arginine metabolic process
small molecule biosynthetic process
NADH dehydrogenase (quinone) activity
organonitrogen compound biosynthetic process doreductase
activity, acting on NAD(P)H, quinone or similar compound as
acceptor NADH dehydrogenase activity NADH dehydrogenase
(ubiquinone) activity
organic acid metabolic process
branched-chain amino acid metabolic process
Number
FDR
Rich factor
D
KEGG pathways annotation
Metabolism
Genetic Information Processes
Environmental Information
Cellular Processes
Organismal Systems
Human Diseases
Number of Unigenes
E
WT
ΔpanD
Z-score
metF
metR
metB
metA
metL
metK
metE
metN
metC
metG
fadB
fadA
fadE
fadI
fadD
fadH
fadL
fadJ
leuO
leuD
leuC
leuA
hisH
hisC
hisF
hisD
hisG
hisS
znuA
znuC
znuB
mglA
mglB
kdpA
kdpB
potG
potF
potH
potI
Metabolism
Methionine
Fatty acid beta oxidation
Leucine
Histidine
Zinc transporter
Galactose transporter
Potassium transporter
Polyamine transporter
F
WT
ΔpanD
cpanD
Relative gene expression
fadH
fadB
fadI
fadD
fadE
metR
hisG
hisF
leuD
leuO
mglA
mglB
kdpA
kdpB
potG
potF
znuA


A
log10(CFU/g liver)
ns
WT
ΔfadAB
ΔmetR
ΔleuO
ΔhisABCDFGHL
ΔkdpABC
ΔmglABC
ΔpotFGHI
B
log10(CFU/g spleen)
C
3dpi body weight (g)
D
P < 0.0001
P = 0.0042
P = 0.0128
P = 0.0307
P = 0.0001
P = 0.0050
ΔznuA
ΔpanD
ΔpanDΔznuA


A
P < 0.0001
P = 0.0001
P < 0.0001
Relative fold replication
1.5
1.0
0.5
0.0
WT
ΔpanD
ΔznuA
ΔpanDΔznuA
B
ns
P = 0.0013
Relative fold replication
1.5
1.0
0.5
0.0
WT
ΔpanD
ΔpanD+100μM ZnSO4
C
P = 0.0001
P = 0.0003
Relative fold replication
1.5
1.0
0.5
0.0
WT
ΔznuA
ΔznuA+1mM β-alanine
D
Macrophage
Zn2+
SCV
SCV
β-alanine
Uptake by Salmonella
Salmonella
Promote Salmonella growth
Dissemination and systemic infection
L-aspartate
PanD
β-alanine
β-alanine
ZnuABC
Zn2+ uptake
Macrophage
SCV (Salmonella-containing vacuole)
Salmonella
Zn2+
Activation
Upregulation