

Salmonella enterica serovar Typhimurium has three transketolase enzymes contributing to the pentose phosphate pathway

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Running title: *Three transketolase isoenzymes in S. Typhimurium*

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ABSTRACT

The genus *Salmonella* is responsible for many illnesses in humans and other vertebrate animals. We report here that *Salmonella enterica* serovar Typhimurium harbors three transketolases that support the non-oxidative branch of the pentose phosphate pathway. BLAST analysis identified two genes, *STM14_2885* and *STM14_2886*, that together encode a putative transketolase (TktC) with 46-47% similarity to the known TktA and TktB isoforms. Assessing the mRNA and protein expression for each of the three transketolases, we determined that all are expressed in wild-type cells and regulated to varying extent by the alternative sigma factor RpoS. Enzyme assays with lysates from wild-type and transketolase-knockout strains established that TktA is responsible for >88% of the transketolase activity in wild-type cells. We purified recombinant forms of each isoenzyme to assess the kinetics for canonical transketolase reactions. TktA and TktB had comparable values for V_{\max} (539-1362 μM NADH consumed $\times \text{sec}^{-1}$), K_M (80-739 μM), and catalytic efficiency (1.02×10^8 - $1.06 \times 10^9 \text{ M}^{-1} \times \text{sec}^{-1}$) for each substrate tested. The recombinant form of TktC had lower K_M values

(23-120 μM), while the V_{\max} (7.8-16 μM NADH consumed $\times \text{sec}^{-1}$) and catalytic efficiency (5.58×10^6 - $6.07 \times 10^8 \text{ M}^{-1} \times \text{sec}^{-1}$) were 10- to 100-fold lower. Using a murine model of *Salmonella* infection, we showed that a strain lacking all three transketolases is avirulent in C57BL/6 mice. These data provide evidence that *S. Typhimurium* possesses three transketolases that contribute to pathogenesis.

The genus *Salmonella* contains more than 2,500 different serovars that are responsible for a range of pathogenic illnesses not only in humans, but in nearly all vertebrate animals (1). In humans, *Salmonella* is one of the most prevalent food-borne pathogens worldwide, causing an estimated 93.8 million infections that result in approximately 155,000 deaths (2). Globally, the most prevalent serovar in humans is *Salmonella enterica* serovar Typhimurium (3). These facultative intracellular bacteria are capable of overcoming host innate immunity using an arsenal of defenses that alter or mitigate the immune response. Many of the defenses are dependent on the reducing power of

NADPH (4-8). Cellular NADPH is generated through the reduction of NADP^+ primarily by enzymes within the pentose phosphate pathway (PPP), a major route of carbohydrate metabolism in species ranging from bacteria and archaea to plants and animals (9-12). The PPP can be divided into an oxidative (oxPPP) and a non-oxidative phase (non-oxPPP). The oxPPP is largely responsible for maintaining cellular redox homeostasis through the reduction of NADP^+ to NADPH, which is critical for *Salmonella* virulence by protecting against innate host defenses (4,5). In the non-oxPPP of *Salmonella*, a series of enzyme-catalyzed reactions generate multiple metabolic intermediates that can be diverted to other pathways, including glycolysis, or serve as precursors for the generation of cellular constituents such as nucleotides, aromatic amino acids, or lipopolysaccharide (LPS) (Figure S1) (11,13). Products from the non-oxPPP can also be channeled back into the oxPPP via gluconeogenesis, allowing carbon to cycle through the pathway. Ultimately, the non-oxPPP allows for dynamic carbon flux to multiple metabolic pathways (14).

Within the non-oxPPP, the dimeric, thiamine pyrophosphate (TPP)-dependent transketolase enzyme catalyzes the rate-limiting step through the reversible transfer of a ketol group to an aldose substrate such as ribose-5-phosphate or erythrose-4-phosphate (15,16). During early studies analyzing variations in bacterial LPS, mutants of *Escherichia coli* or *S. Typhimurium* defective in transketolase activity were isolated (17,18). The *E. coli* transketolase mutants were unable to grow using D-ribose as a sole carbon source, but similar *S. Typhimurium* transketolase mutants would sometimes exhibit slow growth on the same medium. The phenotype in *S. Typhimurium* was not investigated further, but suggests that other transketolases may be encoded by this bacterium. With the development of whole genome sequencing, it is known that the *S. Typhimurium* strain 14028s genome harbors two genes, *tktA* and *tktB*, that encode two transketolase isoenzymes TktA and TktB, respectively (Figure S2 A and B) (19). Herein, a third transketolase isoform in *S. Typhimurium* was identified based on sequence analysis. We present evidence that all three transketolase isoenzymes are functional and contribute to pathogenesis of this bacterium. Furthermore, we determined the regulation,

biological contribution, and kinetic properties of each isoenzyme. Our findings offer deeper insights into the function and regulation of this central metabolic pathway.

RESULTS

Identification and *in silico* analysis of transketolases within Enterobacteriaceae. Aside from the two annotated transketolases in *S. Typhimurium*, two additional genes were found (*STM14_2885* and *STM14_2886*) that, based on BLAST results and sequence analysis, form the components of a transketolase enzyme. *S. Typhimurium* gene *STM14_2886* is located on the complementary strand of the chromosome from 2,505,647 - 2,506,477, and the coding region of *STM14_2885* begins within *STM14_2886* at 2,504,701 - 2,505,654 (Figure S2). Using NCBI Conserved Domains, we mapped out conserved functional regions within each *S. Typhimurium* transketolase and putative transketolase (Figures 1 and S3). Within both TktA and TktB, there were similar conserved dimerization domains in the N-terminal regions, approximately between residues 90 and 210 for each enzyme. The N-termini of TktA and TktB also contained the TPP-binding domain for each enzyme. Analysis of *STM14_2885* and *STM14_2886* revealed that each protein contained both dimerization and TPP binding domains. *STM14_2886* was classified as the N-terminal domain of transketolase containing the catalytic domain, while *STM14_2885* was classified as the C-terminal domain with putative regulatory function. Predictive modeling of each putative transketolase was performed using Swiss-Model to identify conserved structures between the isoenzymes (Figures S4-S6) (20-22). Based on structural similarities between proteins, the sequence analysis results that characterized each putative transketolase as two components of a transketolase enzyme, and to maintain consistent nomenclature between other transketolases, we designated *STM14_2886* as *tktC₁* and *STM14_2885* as *tktC₂*, with gene products TktC₁ and TktC₂, respectively (Figure S2 C and D). We further designated TktC₁ and TktC₂ as TktC when considered together.

The redundancy between the three transketolases in *S. Typhimurium* made us question the similarity between the isoenzymes in *Salmonella* and other Enterobacteriaceae. We made

comparisons between amino acid sequences for each transketolase in *S. Typhimurium*, *E. coli*, and *Salmonella enterica* serovar Typhi, the causative agent of typhoid fever (Tables S3-S5). The sequences for both TktA and TktB showed a high degree of similarity between species and serovars (>96%). The *S. Typhimurium* TktC sequence had the greatest difference, as it was only 47-48% similar to TktA and 41-46% similar to TktB in either *E. coli* or *S. Typhimurium*. After noting the differences between the number of transketolases in *Salmonella* and *E. coli*, we examined the genomes of other Enterobacteriaceae and determined the number of putative transketolase enzymes and transketolase genes (Table S6). Many bacteria in this group possessed only a single transketolase (e.g. *Shigella dysenteriae*), while some had a similar arrangement as *S. Typhimurium* (e.g. *Citrobacter koseri*). Of the organisms that were examined, *Citrobacter rodentium* possessed the greatest number of transketolase genes and putative isoenzymes. However, the reasons for these differences between organisms remain unclear.

Transketolase expression in *S. Typhimurium* shows partial growth phase- and RpoS-regulation. We assessed the expression and regulation of each transketolase gene and isoenzyme in *S. Typhimurium* during different growth phases. *E. coli*, another member of the family Enterobacteriaceae, possesses genes for only two transketolases in its genome (*tktA* and *tktB*), and previous work demonstrated that they are both regulated by the alternative sigma factor RpoS (23). Based on the RpoS-dependent regulation of transketolases in *E. coli*, we compared the transcription and translation of the three *S. Typhimurium* transketolase isoforms in wild-type and *rpoS*-deficient strains (Figure 2). Stationary phase *S. Typhimurium* expressed lower levels of *tktA* mRNA compared logarithmic phase. Although *tktA* was expressed at slightly higher levels in the absence of RpoS, the differences were not statistically significant. While mRNA levels were downregulated during stationary phase, TktA protein expression was unaffected. Expression of *tktB* mRNA and TktB protein in *S. Typhimurium* increased from logarithmic growth to stationary phase, and the upregulation was at least partially dependent on RpoS. Unlike *E. coli* that expresses *tktB* at comparable levels to *tktA* (Figure S7), *tktB*

expression in *S. Typhimurium* was nearly 100-fold lower than *tktA* during logarithmic phase, but only 4-fold lower during stationary phase. Expression of both *tktC*₁ and *tktC*₂ was upregulated during stationary phase in an RpoS-dependent manner. Although mRNA levels of *tktC* were upregulated, protein expression remained unchanged. As anticipated, *katE* and *otsA* followed the known pattern of RpoS regulation (Figure S7) (24-26). Taken together, these data demonstrate that the four genes encoding transketolases and the three protein isoenzymes in *S. Typhimurium* were all expressed under our tested conditions and exhibited varying degrees of growth phase- and RpoS-dependent regulation.

Transketolase-deficient *S. Typhimurium* can grow with glucose, but not ribose, as the sole carbon source. A hallmark of transketolase-deficient *E. coli* is their inability to utilize pentoses as their only source of carbon (27,28). We measured growth kinetics of our library of transketolase mutant *S. Typhimurium* strains in media supplemented with glucose or ribose to determine the role of each of the three putative transketolase isoenzymes in *Salmonella* pentose utilization. Each of the strains grew comparably to wild-type in LB broth (Figure 3 A and D). All strains grew in M9 minimal medium supplemented with 0.2% D-glucose, although the $\Delta tktA \Delta tktB \Delta tktC$ strain had a growth defect compared to wild-type (Figure 3 B and E). In contrast, when grown in M9 minimal medium supplemented with 0.2% D-ribose, the $\Delta tktA$ and $\Delta tktA \Delta tktC$ strains had growth defects (Figure 3 C and F), and the $\Delta tktA \Delta tktB \Delta tktC$ strain had no measurable growth (Figure 3 F). Importantly, when grown in M9 with only D-ribose, *S. Typhimurium* strains lacking two transketolases in any combination (i.e. $\Delta tktA \Delta tktB$, $\Delta tktA \Delta tktC$, or $\Delta tktB \Delta tktC$) exhibited measurable growth, indicating the remaining enzyme in each case could catalyze the transketolase reaction.

Each of the three transketolase isoenzymes in *S. Typhimurium* demonstrate enzymatic activity in cell extracts. To further examine the individual contributions of each transketolase, we measured enzymatic activity in cell extracts derived from transketolase mutant strains (Figures 4 and S8). Cell extracts were obtained from each strain at both logarithmic and stationary phases and used in

transketolase activity assays. The assays were performed with substrates for both canonical transketolase reactions: 1) xylulose-5-phosphate and ribose-5-phosphate (Figure 4) and 2) xylulose-5-phosphate and erythrose-4-phosphate (Figure S8). Loss of *tktA* corresponded with a decrease of approximately 88% of detectable transketolase activity compared to wild-type, suggesting that under the tested conditions, TktA had the largest biological role. However, *S. Typhimurium* strains lacking two transketolases in any combination still yielded detectable enzymatic activity. The resulting activity was attributable to the remaining enzyme in each case, suggesting that while TktA provides the majority of transketolase activity in wild-type cells, all three isoenzymes provide a measurable contribution. The $\Delta tktA \Delta tktB \Delta tktC$ strain yielded no detectable enzymatic activity using xylulose-5-phosphate and ribose-5-phosphate as substrates (Figure 4), confirming that *S. Typhimurium* transketolase activity is encoded by a combination of three transketolases. When using xylulose-5-phosphate and erythrose-4-phosphate as substrates, there was very low NADH consumption detected in the $\Delta tktA \Delta tktB \Delta tktC$ strain cell lysates (Figure S8). This observation is likely due to erythrose-4-phosphate being used as a substrate in combination with residual cellular hexoses by transaldolase (Figure S1). Ultimately, measurable transketolase activity in all except the $\Delta tktA \Delta tktB \Delta tktC$ strain, combined with the inability of this strain to grow using ribose as the sole carbon source (Figure 3), indicates that *S. Typhimurium* possesses three functional, complementary transketolases. Based on these results, we subsequently use Tkt⁰ in reference to the $\Delta tktA \Delta tktB \Delta tktC$ strain.

Ectopic expression of any *S. Typhimurium* transketolase isoenzyme complements Tkt⁰. We constructed pBAD plasmids with wild-type alleles of each transketolase relying on leaky expression from the pBAD promoter. The vectors were transformed into the Tkt⁰ background individually, and the ability of each gene to complement the transketolase deficiency was tested. Each of the complemented strains grew in LB broth without any notable defects (Figure 5A). When growth in M9 minimal medium supplemented with D-glucose was evaluated, the empty vector control plasmid caused a substantial fitness burden on the Tkt⁰ strain, but all strains demonstrated measurable

growth (Figure 5B). The Tkt⁰ strain did not grow in M9 minimal medium supplemented with D-ribose with only the empty vector control, but *in trans* complementation with *tktA*, *tktB*, or *tktC* allowed for growth under the same conditions (Figure 5C). Notably, complementation of the Tkt⁰ strain with individual *tktC* genes, *tktC₁* or *tktC₂*, did not restore growth in M9 minimal media with D-ribose (Figure S9), indicating that both subunits must be expressed for a functional transketolase.

We further evaluated the transketolase activity from the engineered expression vectors by measuring transketolase activity from cell lysates in the Tkt⁰ background (Table S7). There was no detectable activity from the empty vector control or when *tktC₁* or *tktC₂* were expressed individually. There was, however, detectable activity from the Tkt⁰ strain complemented with either *tktA*, *tktB*, or *tktC* when expressed in its wild-type arrangement.

Determining transketolase kinetics from purified recombinant enzyme. After verifying enzymatic activity from recombinant transketolase in the engineered expression vectors *in vivo*, we purified each enzyme. Constructs for TktA and TktB were readily purified to apparent homogeneity (Figure S10) and kinetic parameters were determined using each of the substrates for both canonical transketolase reactions (Table 1, Figures 6 and 7). We were unable to purify the individual constructs for TktC₁ and TktC₂, so we engineered a TktC₁-TktC₂ fusion protein by eliminating the *tktC₁* stop codon and inserting *tktC₂* in-frame immediately downstream. This recombinant form of TktC, denoted here as TktC*, was successfully purified to apparent homogeneity (Figure S10) and kinetic parameters are reported in Table 1 and Figure 8. Protein structural modeling of TktC* revealed a comparable structure to TktC (Figures S6 and S11). TktC* exhibited slightly lower K_M values (23 – 120 μM) compared to TktA and TktB (80 – 730 μM) for each of the tested substrates. Catalytic efficiencies (K_{cat}/K_M) of TktA and TktB were comparable to each other (1.02×10^8 – 1.06×10^9), and were nearly 100 times greater than that of TktC* (5.58×10^6 – 6.07×10^7). Altogether, these results demonstrate that TktC₁ and TktC₂ together form a functional transketolase and provide foundational descriptions of three transketolase isoenzymes in *S. Typhimurium*.

Transketolase-deficient (Tkt⁰) *Salmonella* are avirulent in mice. To determine if transketolases are involved in *S. Typhimurium* pathogenesis, the virulence of the Tkt⁰ strain in C57BL/6 mice was assessed (Figure 9). Intraperitoneal inoculation of Tkt⁰ *S. Typhimurium* was not lethal to the mice and animals did not exhibit any clinical signs of disease. These findings suggest that lack of transketolases and disruption of the non-oxPPP attenuates *S. Typhimurium*.

DISCUSSION

Data from the above experiments deliver multiple lines of evidence that *S. Typhimurium* possesses three functional transketolase isoenzymes, each with unique features and characteristics. Prior work has clearly demonstrated that regulation of *E. coli* transketolases is dependent on both growth phase and the alternative sigma factor RpoS (23,29-31). We observed a similar pattern of regulation in *S. Typhimurium*, most notably transcriptionally and translationally for TktB, and transcriptionally for both genes of TktC. Interestingly, protein levels for all three transketolases in *S. Typhimurium* were at or near their highest expression during stationary phase. During stationary phase in gram-negative bacteria, starvation conditions trigger RpoS, which subsequently promotes expression of genes that prepare the cell for survival (32-34). Additionally, RpoS induces cross-protection against diverse stresses, including osmotic, temperature, acid, oxidative, and nitrosative (4,35-39). The contribution of RpoS to combatting stressful conditions is linked to its essential role in *Salmonella* virulence (40). Although a direct role for transketolases in virulence or resisting stress conditions has not been described previously, our results demonstrate that TktB and TktC are a part of the RpoS regulon, indicating that they may have a part in these cellular functions. The ability of each isoenzyme to catalyze the transketolase reaction highlights the overlapping and complementary functions of each enzyme; however, the differences in expression and enzyme kinetics suggest that each *S. Typhimurium* transketolase may have a unique role in this bacterium.

The reasons underlying why *S. Typhimurium* maintains three transketolases remain unclear. However, given that *S. Typhimurium* transits through varied and stressful environments, we

predict that the individual transketolases in *S. Typhimurium* will each make unique contributions to cellular physiology. Even though TktA is responsible for the majority of transketolase activity in *S. Typhimurium*, both TktB and TktC are independently sufficient to allow growth under laboratory conditions. A similar phenomenon has been observed in *E. coli* where up to 90% of transketolase activity was attributable to TktA (41). In the same study, a $\Delta tktA$ mutant demonstrated slow growth on MacConkey agar containing ribose while $\Delta tktA \Delta tktB$ mutant showed no growth. Similarly, our study demonstrated that in the *S. Typhimurium* Tkt⁰ strain complemented with *tktC*, even the low levels of transketolase activity were sufficient to allow growth in minimal medium with ribose as the sole carbon source (Table S7 and Figure 5C). In other species for which transketolase kinetic constants have been determined, k_{cat} values can be similar to ten-fold lower, as is the case with *Saccharomyces cerevisiae*, or more than three orders of magnitude lower as in *Bacillus methanolicus* (42,43). These observations raise questions about the minimal level of transketolase activity required for growth and why wild-type *S. Typhimurium* utilizes three transketolase isoenzymes that exhibit high levels of transketolase activity. One possible answer to these questions is that transketolases are necessary for conditions other than routine growth, and each transketolase has unique properties. Our data indicate that transketolases are required for *S. Typhimurium* virulence, but polar effects on other virulence pathways resulting from transketolase dysregulation and the contributions of individual transketolases remains to be tested. Even though TktA and TktB are 75% identical and 86% similar, these two transketolases are differentially regulated by growth phase and RpoS. It is plausible that these two transketolases have their main effects during different growth phases or that TktB may be more beneficial during stressful conditions encountered during infection. An additional possibility is that TktA may be the most efficient enzyme and responsible for routine growth, but it may be more susceptible to oxidative and/or nitrosative stresses than the other isoenzymes, TktB and/or TktC. Such a situation is not without precedent among bacterial metabolic isoenzymes, as the two aconitase enzymes of *E. coli*, AcnA and AcnB, demonstrate opposing sensitivities to oxidative stress (44). It has

also been shown that in *Arabidopsis thaliana*, transketolase and ribose-5-phosphate isomerase, both of which are enzymes of the non-oxPPP, are susceptible to nitration and nitrosylation (45-47). Additionally, abundance of these enzymes increases when cellular levels of S-nitrosylation increase. *Saccharomyces cerevisiae* mutants deficient in transaldolase, another enzyme of the non-oxPPP, were more sensitive to oxidative stress in a mechanism that appeared to be independent of NADPH, highlighting the importance of the non-oxPPP during stressful conditions (48).

S. Typhimurium transketolases are intriguing not only because the organism possesses three functional isoenzymes, but also because the third transketolase, TktC, is the product of two genes. This is, to our knowledge, the first time such a transketolase enzyme has been analyzed in prokaryotes. BLAST analysis did not identify any homologues of TktC in prokaryotes that were encoded by a single gene; however, the recombinant form of TktC engineered with the two genes fused together produced a functional and efficient transketolase. The observation that *S. Typhimurium* and a wide variety of other Enterobacteriaceae have maintained the two-gene arrangement suggests that this organization confers some advantage to the organisms. In *S. Typhimurium*, the two genes for TktC are highly divergent from both *tktA* or *tktB*, and even from *STY2570-STY2571* in *S. Typhi* (Tables S3-S5), but the reasons underling this genetic arrangement are unclear. By deducing the specific conditions under which TktC is most necessary and useful to the organism, we hope to reveal clues regarding why *Salmonella* and other organisms have this arrangement of transketolase isoenzymes. Ultimately, this study provides evidence for three transketolase isoenzymes that contribute to pathogenesis of *S. Typhimurium*, each with their own unique properties and characteristics. The results offer a foundation for future investigations into the functions of transketolases and other enzymes of the PPP.

EXPERIMENTAL PROCEDURES

Classification of Transketolase Domains

The NCBI-ProteinID for each *S. Typhimurium* transketolase (TktA, ACY90124; TktB, ACY89466; STM14_2886/TktC₁, ACY89325; STM14_2885/TktC₂, ACY89324) was used as the

query sequence to search the NCBI Protein database. Each sequence was then subjected to analysis to identify conserved domains (49-52). Specific residues predicted to be involved in dimerization and TPP binding were also classified this way.

Bacterial Strains and Plasmids

S. enterica serovar Typhimurium strain ATCC 14028s was used as the wild-type strain and as the background for mutant construction for this study. Mutant strains (Table S1) were created following the method described by Datsenko and Wanner (53). Primers with homologous extensions that corresponded to the gene to be knocked out were used to amplify a kanamycin resistance gene flanked by flippase recognition targets (FRT) from the plasmid pKD13 using TaKaRa LA Taq high-fidelity polymerase (Fisher Scientific, Pittsburgh, PA) (Table S2). The resulting product was purified and concentrated using Thermo Scientific GeneJET PCR Purification system (Thermo Fisher, Waltham, MA) and was transformed into a competent *S. Typhimurium* strain TT22236 containing the plasmid pTP223 that expresses an isopropyl- β -thiogalactopyranoside (IPTG)-inducible λ -Red recombinase enzyme. Mutations were transduced into wild-type *S. Typhimurium* using P22 phage. Pseudolysogens were eliminated via streaking of colonies on Evans blue uranine agar to screen for phage-free cells. Excision of the kanamycin resistance cassette was achieved by electroporating strains with the temperature-sensitive plasmid pCP20, which encodes a FLP recombinase. The FLP recombinase catalyzes recombination of the FRT sites flanking the kanamycin resistance cassette, thereby removing the cassette and leaving a single FRT scar. PCR analysis using primers outside of the gene of interest was used to confirm deletion of the gene.

Complementation and expression vectors were engineered in pBAD plasmids using the specified primers (Table S2). Wild-type alleles of genes were inserted into the pBAD multiple cloning site using indicated restriction enzymes and subsequent ligation. Resulting constructs were under the control of an arabinose-inducible pBAD promoter with an N-terminal 6x-His tag. A *tktC₁-tktC₂* fusion without overlapping genes was created using seamless cloning (Invitrogen, Carlsbad, CA). The start and stop codons were removed from both *tktC₁*

and *tktC*₂, and *tktC*₂ was moved to be in-frame immediately downstream of *tktC*₁ (Table S2). The resulting plasmid construct, designated pBAD::*tktC**, consisted of the fused genes under an arabinose-inducible promoter with an N-terminal 6x-His tag.

Bacterial Growth

Strains were inoculated from frozen bacterial stocks and grown to stationary phase overnight (20 hours) in LB broth at 37°C with constant agitation. Overnight cultures of strains were diluted 1:500 or 1:250 (as indicated) into fresh LB or M9 minimal medium supplemented with 0.2% D-glucose or 0.2% D-ribose, and optical density was determined by measuring absorbance at 600 nm (OD₆₀₀) every 15 minutes while incubated at 37°C with constant agitation in a Cytation 5 microplate reader (Biotek, Winooski, VT). Blank values for each condition were obtained by subtracting the absorbance at OD₆₀₀ of media alone from the absorbance at OD₆₀₀ of the bacterial cultures. For gene and protein expression analysis and for transketolase activity assays, overnight cultures were subcultured 1:500 into fresh LB and grown at 37°C with constant agitation to mid-logarithmic phase (OD₆₀₀ = 0.5) or stationary phase (20 h).

Gene Expression

Gene expression was measured in three or four independent samples from wild-type and $\Delta rpoS::FRT$ *S. Typhimurium* at logarithmic and stationary phases. At the indicated time point, 1.5 mL of bacterial culture was harvested by centrifugation for 1 minute at 12,000 x g. Culture supernatant was removed and the cell pellet was resuspended in 0.5 mL RNazol (Molecular Research Center, Inc., Cincinnati, OH) and processed following manufacturer's protocol for total RNA isolation. DNA was depleted from samples using Ambion TurboFree DNase (Thermo Fisher) and RNA was purified using GeneJet RNA Purification and Concentration Mini Kit (ThermoFisher). RNA was quantified spectrophotometrically using the Take3 application and Cytation 5 microplate reader (BioTek), and 1.5 mg of each sample was reverse transcribed using the High Capacity cDNA Synthesis kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. An equal amount of sample was diluted in buffer for no reverse

transcriptase controls. Two technical replicates of each sample were analyzed by qRT-PCR (25 ng per reaction, total volume 20 μ L) with Bullseye EvaGreen Master Mix (MidSci, St. Louis, MO) using the CFX Connect (BioRad, Hercules, CA) with the following cycling parameters: 1) 95°C, 10 min; 2) 95°C, 3s; 60°C 30s; Repeat 40x. Dilutions of *S. Typhimurium* gDNA were used to measure primer efficiency for each gene that was assessed, and all sets had greater than 97% efficiency (Tables 2 and S2). RNA quality was assured by comparing Ct values for primers at two locations in the 5' region of *rpoD*. Amplification specificity was determined by melt curve analysis following qRT-PCR. Relative expression of each gene was calculated as relative to the housekeeping sigma factor *rpoD* using the $2^{-\Delta\Delta C_t}$ method (54).

Western Blotting

Salmonella strains grown to logarithmic or stationary phase in LB broth were harvested by centrifuging 1 mL of cells at 12,000 x g for 1 minute and resuspending the pellet in 500 μ L lysis buffer (0.1M Tris, 4% SDS, pH=7.8). Samples for TktA::FLAG expression analysis were diluted 1:10 due to high expression, but representative western blot images show undiluted samples. All samples were immediately boiled in sample loading buffer (BioRad) and denatured proteins were separated on an Any kD Mini-PROTEAN TGX Precast protein gel (BioRad). Equal loading of each cell lysate was determined using the BCA assay kit with bovine serum albumin as a standard (Fisher Scientific) and stain-free gel assessment before blotting proteins to a PVDF membrane. Membranes were blocked with 5% milk in TBST for 2 hours, washed, incubated 2 hours with mouse α -FLAG antibody (Invitrogen, Catalog No. MA121315, Lot No. RK241385) diluted 1:1000 in TBST, washed, incubated 2 hours with goat α -mouse-HRP (Invitrogen, Catalog No. 31460, Lot No. SA245916) diluted 1:2000 in TBST, washed, and imaged using Western Sure Premium ECL (Licor, Lincoln, NE) with a ChemiDoc MP Gel Imaging System (BioRad). Band intensity for each lane was determined using ImageLab software and local subtraction algorithms (BioRad).

Transketolase Activity Assays

Cells were grown to logarithmic or stationary phase as described above with 100 μ g/mL

ampicillin and 100 μ M IPTG (Fisher Scientific) when appropriate. For logarithmic and stationary phase cells, 1 mL and 100 μ L of culture was harvested, respectively. Cells were washed in 1 mL PBS, pelleted by centrifugation, and resuspended in 500 μ L 50 mM Tris, pH=7.5. Samples were freeze-thawed at -75°C , then sonicated on dry ice (5 s on, 10 s off, repeat 4 times). Samples were centrifuged at $12,000 \times g$ at 4°C for 10 min to remove cell debris. Supernatants were used for the assay as previously described (55) (Figure S12). Briefly, protein concentration was determined using the BCA assay kit with bovine serum albumin as a standard (Fisher Scientific). For the enzymatic reactions, each well contained a final volume of 100 μ L consisting of 50 mM Tris (pH=7.5), 0.24 mM MgCl_2 , 10 μ M thiamine pyrophosphate, 0.5 mM NADH, 3 U of glycerol 3-phosphate dehydrogenase, 10 U of triosephosphate isomerase, 0.5 mM D-xylulose-5-phosphate, and 0.5 mM D-ribose-5-phosphate or D-erythrose-4-phosphate (all reagents obtained from Sigma Aldrich, St. Louis, MO). The reaction proceeded at 37°C and the decrease in $A_{340\text{ nm}}$ was monitored with a Cytation 5 microplate reader (BioTek). One unit of enzyme was defined as the amount of enzyme that oxidized 1 μ mol of NADH per minute. Transketolase enzyme activity for cell lysates was expressed as units per milligram total protein. Limits of detection (LOD) and quantification (LOQ) were determined from all replicate blank readings for each assay (56,57). LOD was calculated as mean plus three standard deviations; LOQ was calculated as mean plus ten standard deviations.

Purified transketolase enzyme kinetics were measured using the same reaction as above, with the exception that one sugar substrate was present in excess while the other sugar co-substrate was present at the concentration indicated (Figures 6-8). Reactions containing excess D-xylulose-5-phosphate had a concentration of 2 mM, reactions containing excess D-ribose-5-phosphate had a concentration of 10 mM, and reactions containing excess D-erythrose-4-phosphate had a concentration of 1 mM (all materials were obtained as described above, with the exception of D-xylulose-5-phosphate which was purchased from Carbosynth, Compton, Berkshire, United Kingdom). Enzymatic activity for each reaction was calculated as μ M NADH consumed per second.

Enzyme Purification

Under physiological conditions, transketolases dimerize to form functional enzymes (16) and because of the high similarity between *S. Typhimurium* transketolase isoenzymes, we purified recombinant protein from the Tkt⁰ ($\Delta tktA \Delta tktB \Delta tktC$) background to eliminate the possibility of heterodimer formation and co-purification. Overnight cultures were subcultured 1:500 into fresh LB with ampicillin and allowed to grow to $\text{OD}_{600}=0.5$, at which time arabinose was added to a final concentration of 0.2%. Strains were grown for an additional 4 hours at which time 50 mL of culture was harvested via centrifugation. Supernatants were removed, and cell pellets were resuspended in 10 mL equilibration/wash buffer (50mM sodium phosphate, 300mM sodium chloride, 10mM imidazole, pH=7.4). For TktC* purification, equilibration/wash buffer also contained Protease Inhibitor Cocktail (Sigma Aldrich). Cell suspensions underwent 5 freeze-thaw cycles from -80°C to 37°C . Lysozyme (Fisher Scientific) was added to each sample during thaw of cycle 4. After last thaw, samples were incubated on dry ice while sonication was performed with the following conditions: 30 s on, 30 s off, repeat 6x beginning at 50% and increasing by 10% each cycle. Cell debris was removed by centrifugation at $3,000 \times g$ for 30 minutes at 4°C . Supernatant was then added to 3 mL HisPur Cobalt Spin Columns (Thermo Fisher) and the manufacturer's protocol was followed for purification of His-tagged proteins using native buffer conditions, with the exception that purified protein was eluted using an increasing gradient of imidazole (10 mM, 25 mM, 50 mM, 75 mM, 100 mM, 150 mM, and 200 mM). The protein from the final elution was used for all subsequent assays. Protein concentration was determined by BCA assay using bovine serum albumin as a standard (Fisher Scientific). Purity of each transketolase was determined by loading 150-250 μ g of each sample into an Any kD Mini-PROTEAN TGX Stain-Free Gel (BioRad) and analyzing the lane using ImageLab software (BioRad).

Mouse Infections

Eight-week-old C57BL/6 mice bred in the University of Colorado School of Medicine animal facility according to Institutional Animal Care and Use Committee guidelines were used to assess the

role of transketolases in *Salmonella* pathogenesis. Individual animals were inoculated intraperitoneally with approximately 200 CFU of *Salmonella* grown overnight to stationary phase in LB broth. Mouse survival was monitored for three weeks. Mice manifesting signs of distress (e.g., low spontaneous activity and ruffled coat) were humanely euthanized by CO₂ inhalation followed by cervical dislocation.

Statistical Analysis

Data from gene and protein expression, as well as transketolase activity from cell lysates, were analyzed using GraphPad Prism (version 6.01) with one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Results were considered statistically significant (*) when $p < 0.05$. Enzyme kinetics were determined using GraphPad Prism

(version 6.01) with Michaelis-Menten least squares (ordinary) fit method.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article

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FOOTNOTE

Abbreviations used: PPP, pentose phosphate pathway; oxPPP, oxidative pentose phosphate pathway; non-oxPPP, non-oxidative pentose phosphate pathway; LPS, lipopolysaccharide; TPP, thiamine pyrophosphate

Table 1. Kinetic analysis of *S. Typhimurium* transketolases.

Reaction Substrates ¹	V_{\max} ($\mu\text{M NADH}$ consumed $\times \text{sec}^{-1}$)	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M ($\text{M}^{-1} \text{sec}^{-1}$)
Transketolase A				
<u>Excess X5P</u>				
R5P	992 ± 60	730 ± 110	9.54×10^4	1.31×10^8
E4P	1079 ± 97	99 ± 20	1.04×10^5	1.05×10^9
<u>Excess R5P</u>				
X5P	1362 ± 42	270 ± 30	1.31×10^5	4.77×10^8
<u>Excess E4P</u>				
X5P	743 ± 36	170 ± 30	7.14×10^4	4.22×10^8
Transketolase B				
<u>Excess X5P</u>				
R5P	690 ± 25	660 ± 60	6.71×10^4	1.02×10^8
E4P	888 ± 57	82 ± 10	8.65×10^4	1.06×10^9
<u>Excess R5P</u>				
X5P	806 ± 25	290 ± 30	7.85×10^4	2.69×10^8
<u>Excess E4P</u>				
X5P	539 ± 33	180 ± 40	5.25×10^4	2.99×10^8
Transketolase C*				
<u>Excess X5P</u>				
R5P	15.5 ± 1.1	58 ± 14	1.35×10^3	2.32×10^7
E4P	16.0 ± 0.7	23 ± 4	1.39×10^3	6.07×10^7
<u>Excess R5P</u>				
X5P	9.4 ± 0.5	75 ± 18	8.16×10^2	1.08×10^7
<u>Excess E4P</u>				
X5P	7.8 ± 0.3	120 ± 15	6.71×10^2	5.58×10^6

¹Kinetics of each purified enzyme were analyzed for each of the canonical transketolase reactions. Each substrate was assessed while the co-substrate was present in excess. Values for V_{\max} and K_M represent mean and standard deviation. X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate.

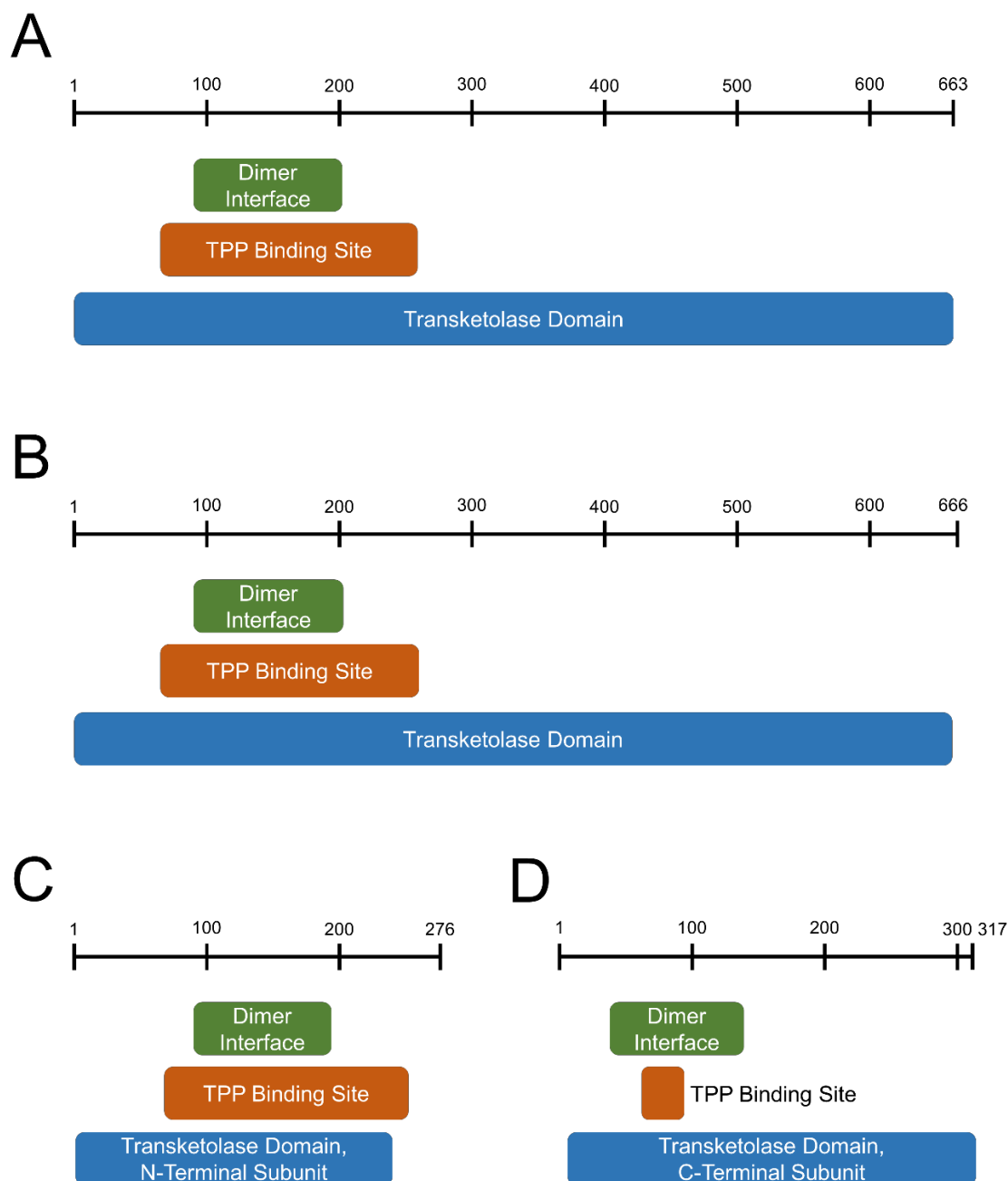


Figure 1. Conserved domains of *S. Typhimurium* transketolases. Representations of amino acid sequences are shown for **A)** TktA, **B)** TktB, **C)** TktC₁, and **D)** TktC₂. Regions of the sequence that were identified as a conserved domain are shown below the corresponding residue numbers.

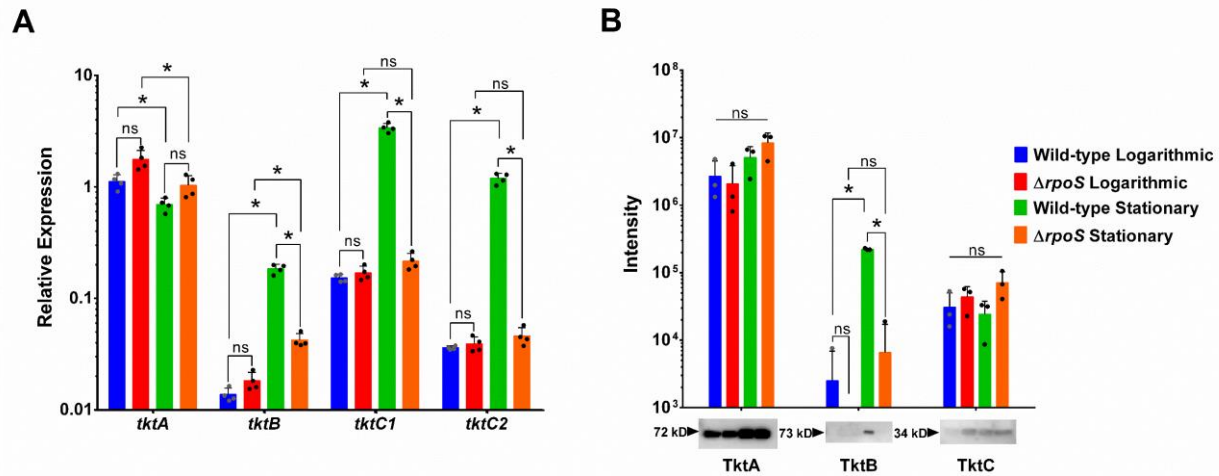


Figure 2. Transketolase expression is partially growth phase- and RpoS-regulated. Overnight cultures grown in LB were diluted 1:500 into fresh LB and cells were harvested from logarithmic ($OD_{600} = 0.5$) or stationary phase ($t = 20$ h) cultures. **A)** Transcription of the four transketolase genes in *S. Typhimurium* was evaluated in wild-type and *rpoS* mutant bacteria using qRT-PCR. mRNA levels were normalized to the housekeeping sigma factor *rpoD*. Expression is represented as mean and standard deviation of six or eight measurements from three or four independent biological replicates with histograms and error bars. Individual data points for each biological replicate are represented as circles and overlaid on each histogram. **B)** Translational expression of each FLAG-tagged transketolase in *S. Typhimurium* was measured by western blot analysis. Expression is represented as mean intensity and standard deviation of each protein band with histograms and error bars. Data represent measurements from three independent experiments and individual data points are overlaid on each histogram. Representative western blots are depicted below the summary data, and each band corresponds to the bar directly above. Data in A and B were analyzed by one-way ANOVA followed by Tukey's post-hoc test. Differences were considered significant (*) if $p < 0.05$. (ns, not significant).

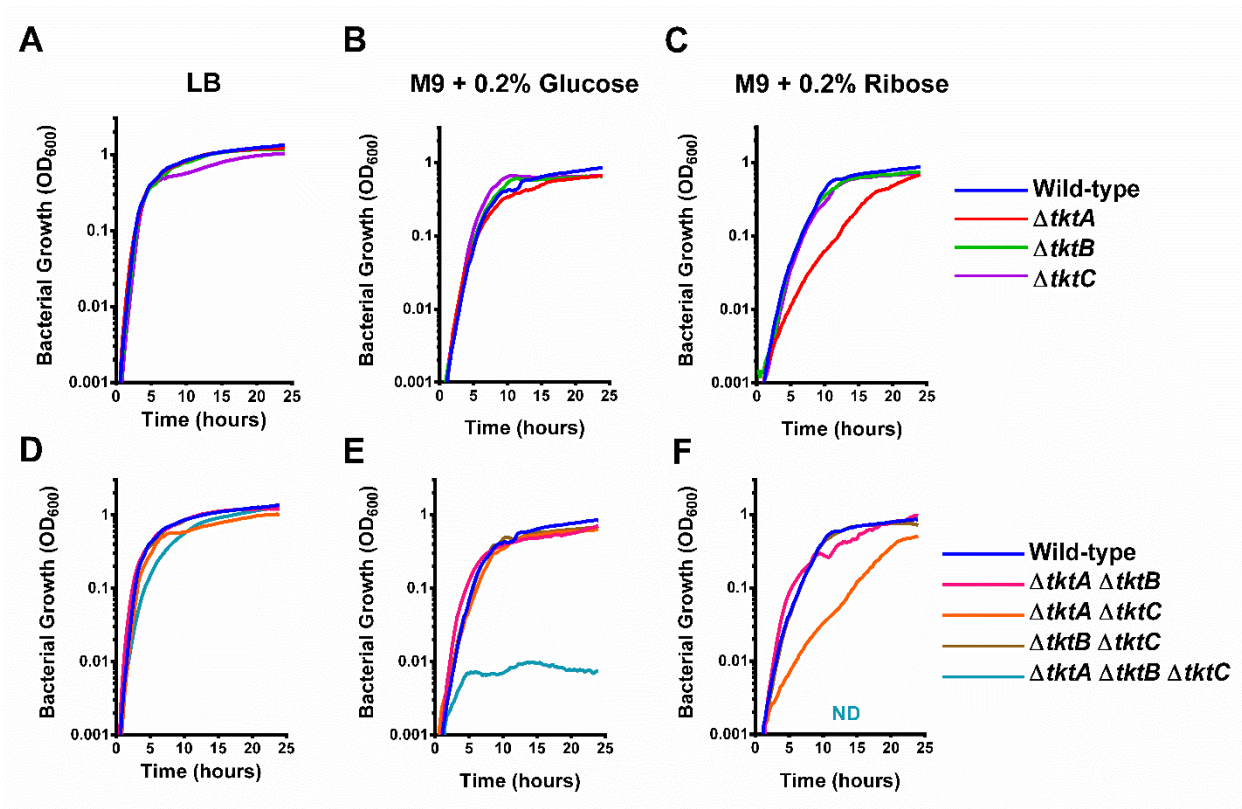


Figure 3. Growth kinetics of *S. Typhimurium* transketolase mutants. Overnight cultures of each strain grown in LB were diluted 1:1000 into fresh LB (**A and D**), M9 minimal medium supplemented with 0.2% D-glucose (**B and E**), or M9 minimal medium supplemented with 0.2% D-ribose (**C and F**). Cultures were incubated in 96-well plates at 37°C with constant agitation. OD₆₀₀ of each culture was measured every 15 minutes. Data represent means of 4-6 measurements from 3 independent experiments. ND, growth was not detected

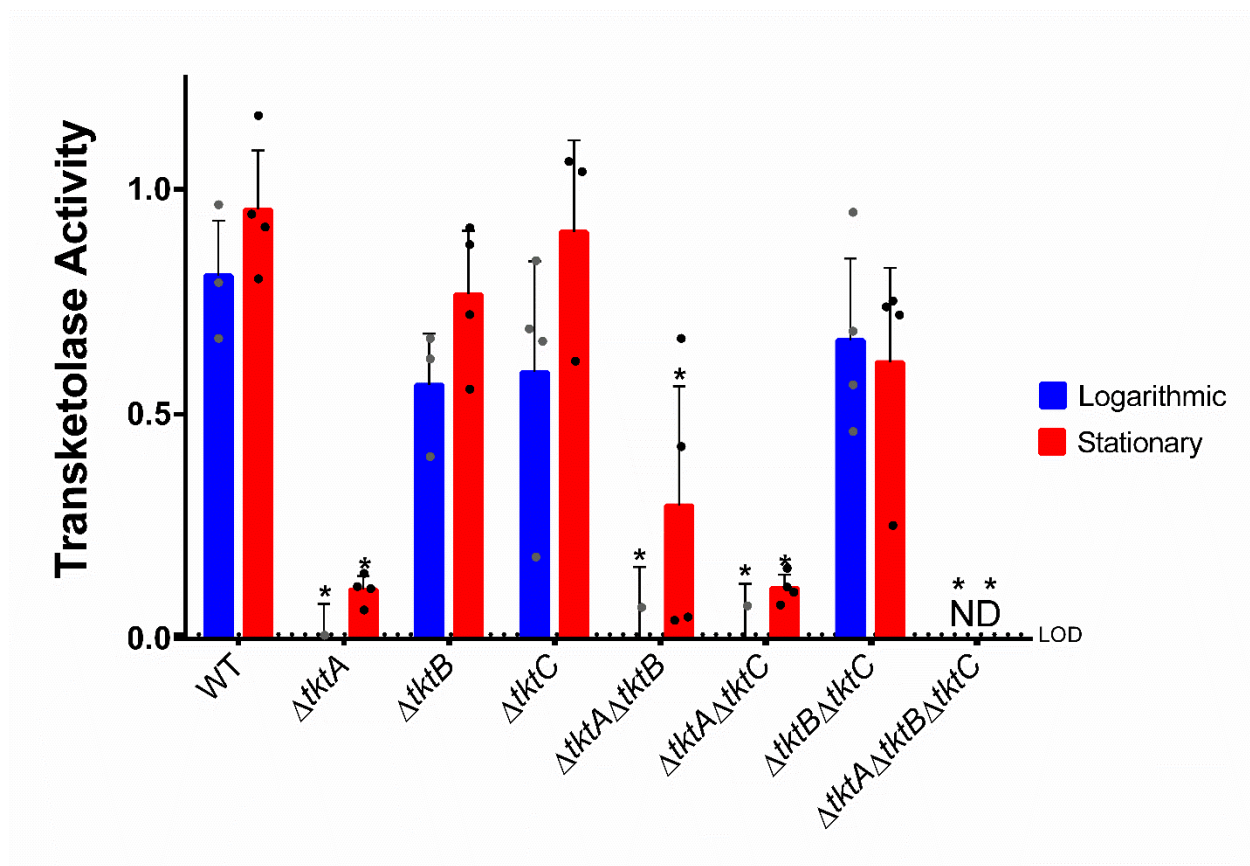


Figure 4. Transketolase activity in *S. Typhimurium*. Transketolase enzymatic activity was measured from cell extracts of transketolase knockout strains harvested at either logarithmic ($OD_{600} = 0.5$) or stationary ($t = 20$ h) phase. The assay was performed using xylulose-5-phosphate and ribose-5-phosphate as substrates. Transketolase activity is expressed as units per milligram of total protein input, where one unit is defined as the amount of enzyme that oxidized $1 \mu\text{mol}$ of NADH per minute. Histograms and error bars represent means and standard deviations of 3 or 4 independent experiments. Individual data points are overlaid on each histogram. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test comparing each knockout strain to wild-type at the same growth phase. Differences were considered significant (*) if $p < 0.05$. Limit of detection (LOD) for the assay was 0.002.

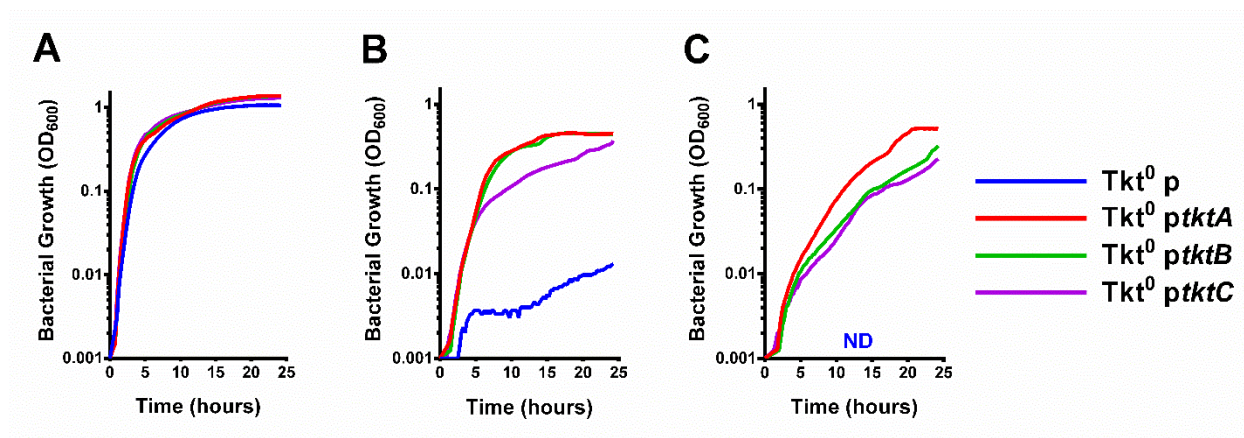


Figure 5. Growth kinetics of Tkt⁰ *S. Typhimurium* complemented with wild-type transketolase alleles. Overnight cultures of each strain grown in LB were diluted 1:250 into fresh LB (A), M9 minimal medium supplemented with 0.2% D-glucose (B), or M9 minimal medium supplemented with 0.2% D-ribose (C). Cultures were incubated in 96-well plates at 37°C with constant agitation. OD₆₀₀ of each culture was measured every 15 minutes. Data represent means of 4 independent experiments. ND, growth was not detected.

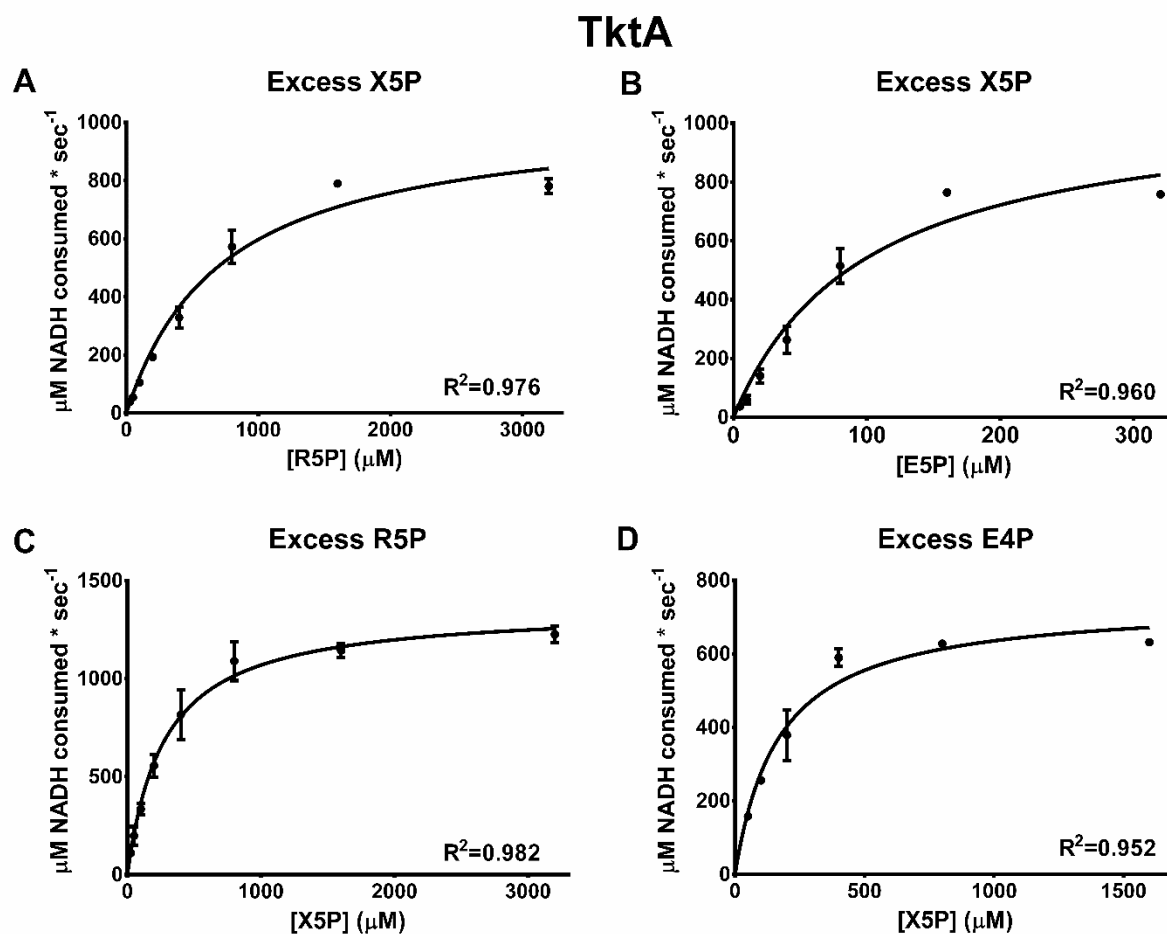


Figure 6. Saturation curves for purified transketolase A using canonical transketolase substrates. Enzymatic activity for each reaction condition was determined and represented as $\mu\text{M NADH consumed per second}$. Data for each reaction condition represent mean and standard deviation for a single measurement from two independent experiments. R^2 values for each best-fit line appear in the lower right corner of each graph. Detailed values from these experiments are shown in Table 1. X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate.

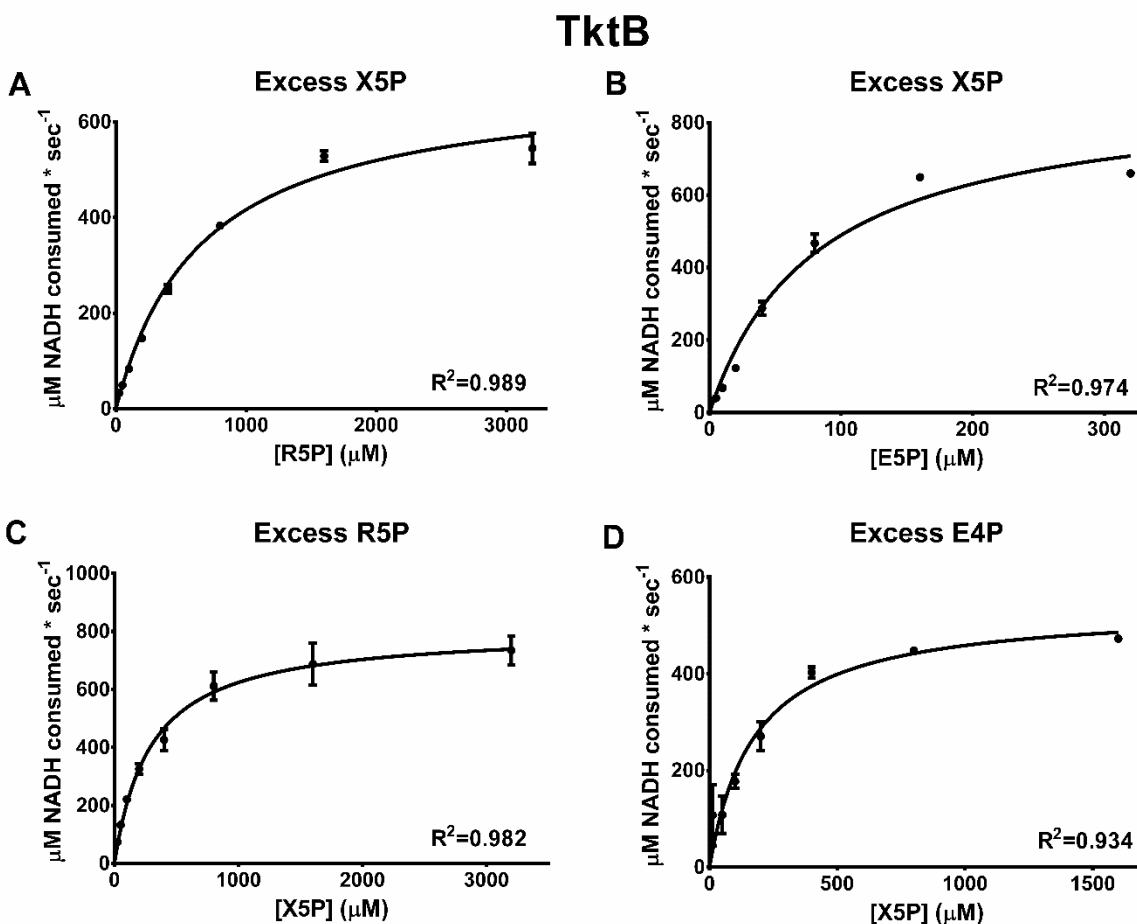


Figure 7. Saturation curves for purified transketolase B using canonical transketolase substrates. Enzymatic activity for each reaction condition was determined and represented as $\mu\text{M NADH consumed per second}$. Data for each reaction condition represent mean and standard deviation for a single measurement from two independent experiments. R^2 values for each best-fit line appear in the lower right corner of each graph. Detailed values from these experiments are shown in Table 1. X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate.

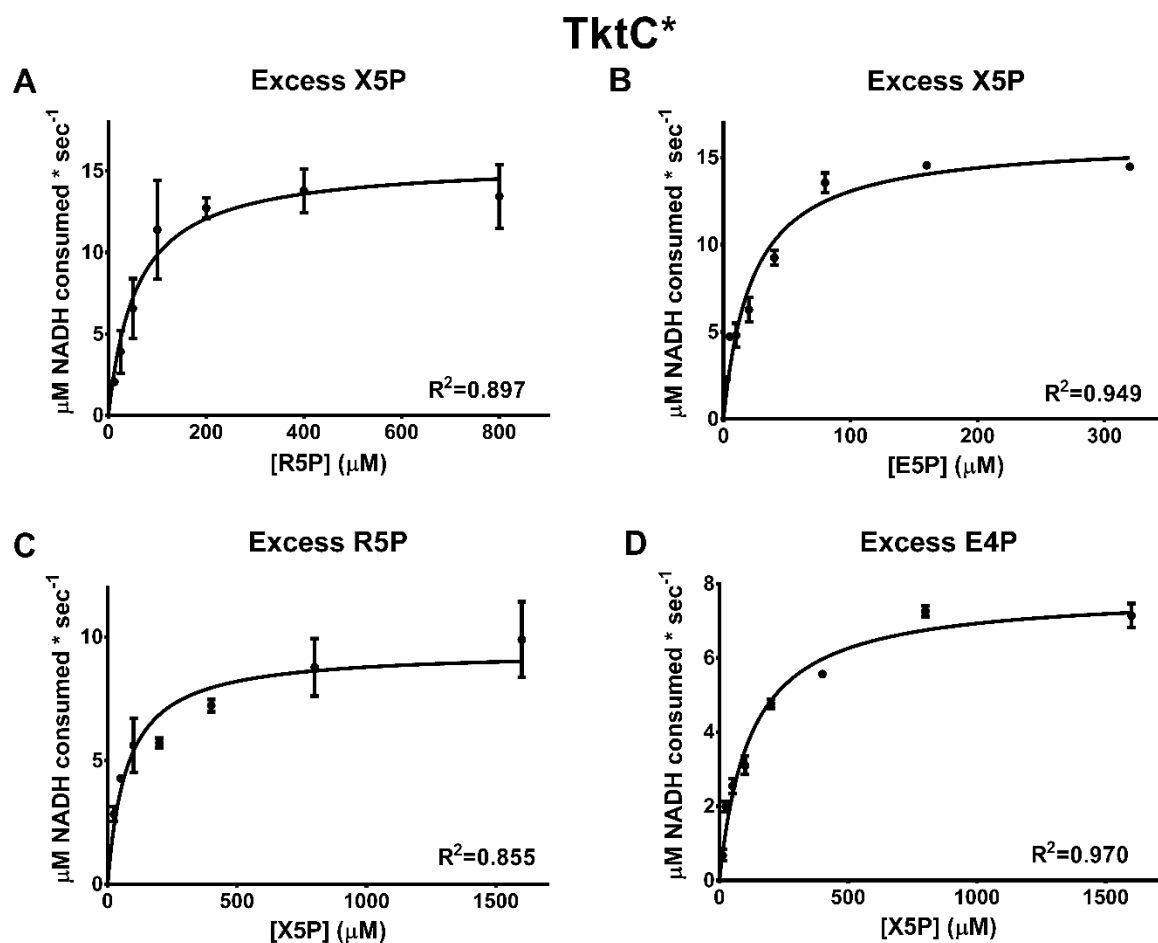


Figure 8. Saturation curves for purified transketolase C* using canonical transketolase substrates. Enzymatic activity for each reaction condition was determined and represented as $\mu\text{M NADH consumed per second}$. Data for each reaction condition represent mean and standard deviation for a single measurement from two independent experiments. R^2 values for each best-fit line appear in the lower right corner of each graph. Detailed values from these experiments are shown in Table 1. X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate.

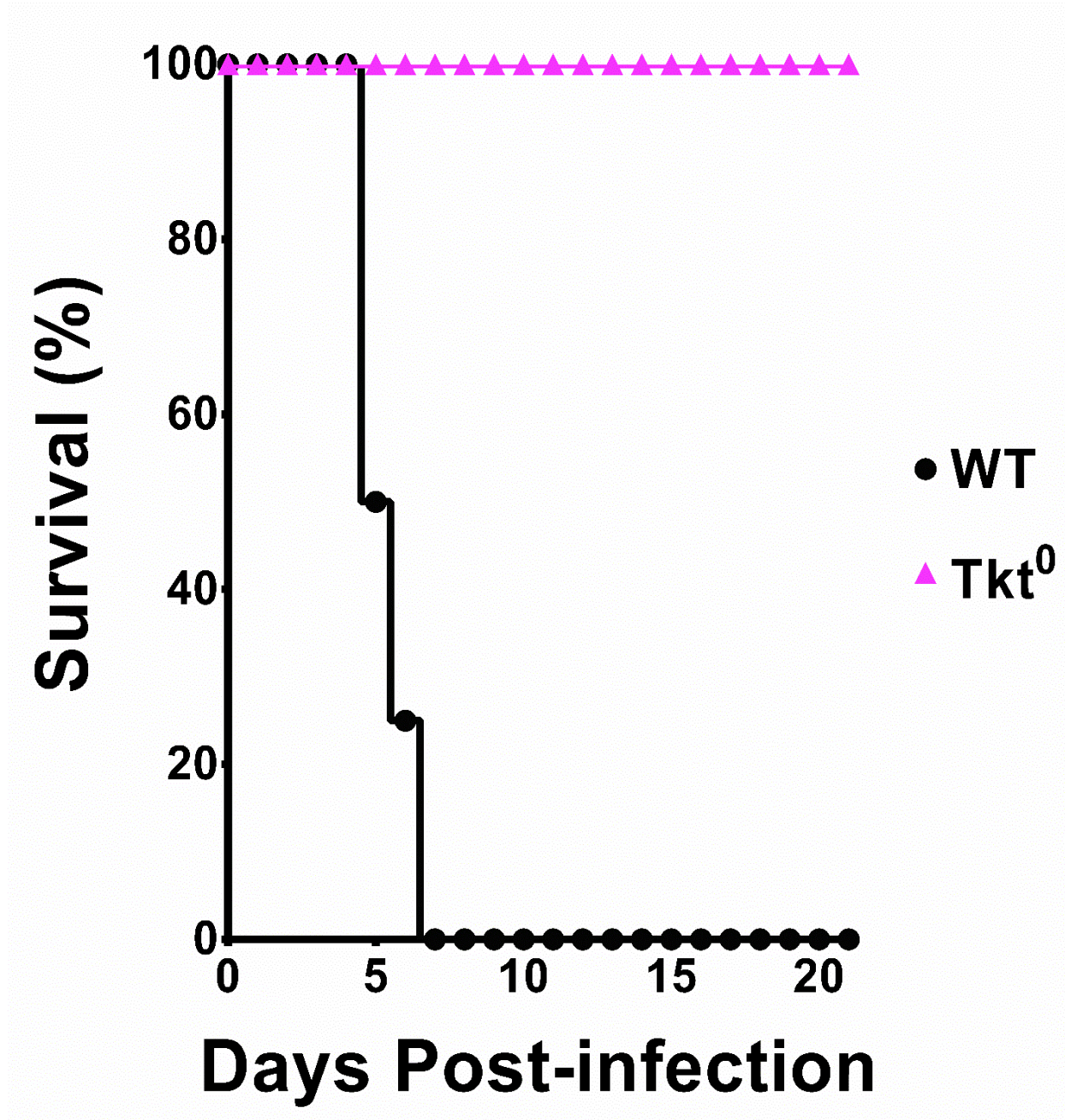


Figure 9. Transketolase-deficient (Tkt⁰) *Salmonella* are avirulent in mice. C57BL/6 mice were inoculated intraperitoneally with ~200 CFU of either wild-type or $\Delta tktA \Delta tktB \Delta tktC$ *S. Typhimurium*. The percentage of mice surviving the experimental *Salmonella* infections were evaluated over time. Data represent five mice for the group infected with Tkt⁰ and four mice for the group infected with wild-type.

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