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Integration of the pSLT Plasmid into the *Salmonella* Chromosome Results in a Temperature-Sensitive Growth Defect Due to Aberrant DNA Replication

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ABSTRACT A mutant of *Salmonella enterica* serovar Typhimurium was isolated that simultaneously affected two metabolic pathways as follows: NAD metabolism and DNA repair. The mutant was isolated as resistant to a nicotinamide analog and as temperature-sensitive for growth on minimal glucose medium. In this mutant, *Salmonella's* 94-kb virulence plasmid pSLT had recombined into the chromosome upstream of the NAD salvage pathway gene *pncA*. This insertion blocked most transcription of *pncA*, which reduced uptake of the nicotinamide analog. The pSLT insertion mutant also exhibited phenotypes associated with induction of the SOS DNA repair system, including an increase in filamentous cells, higher exonuclease III and catalase activities, and derepression of SOS gene expression. Genome sequencing revealed increased read coverage extending out from the site of pSLT insertion. The two pSLT replication origins are likely initiating replication of the chromosome near the normal replication terminus. Too much replication initiation at the wrong site is probably causing the observed growth defects. Accordingly, deletion of both pSLT replication origins restored growth at higher temperatures.

IMPORTANCE In studies that insert a second replication origin into the chromosome, both origins are typically active at the same time. In contrast, the integrated pSLT plasmid initiated replication in stationary phase after normal chromosomal replication had finished. The gradient in read coverage extending out from a single site could be a simple but powerful tool for studying replication and detecting chromosomal rearrangements. This technique may be of particular value when a genome has been sequenced for the first time to verify correct assembly.

KEYWORDS AspC, DNA replication, NAD metabolism, TyrB, integrated plasmid

NAD and NADP and their reduced forms, NADH and NADPH, are the major donors and acceptors of electrons in cellular metabolism. In addition, NAD cleavage serves as the energy source for DNA ligation in bacteria (1, 2). *Salmonella* produces NAD either *de novo* from aspartate and dihydroxyacetone phosphate or from exogenous pyridine sources like nicotinamide (Nm) and nicotinic acid (NA) (Fig. 1). Nm is taken up by cells and then converted to NA by nicotinamide deamidase (PncA). NA is converted to the NAD biosynthetic pathway intermediate NaMN by nicotinate phosphoribosyltransferase (PncB). *Salmonella* also generates Nm, NA, and NaMN by recycling the nicotinamide mononucleotide (NMN) produced by DNA ligase when NAD is cleaved (3, 4).

Mutations in the NAD pathways can be isolated using the bacteriostatic 6-amino analogs of Nm and NA as follows: 6-aminonicotinamide (6ANm) and 6-aminonicotinic acid (6ANA). These analogs are taken up and converted to 6-amino-NAD and 6-amino-NADP. Mutants resistant to the 6-amino analogs most often have a mutation in either *pncA* or *pncB* that prevents the analogs from entering NAD metabolism (5). Null alleles

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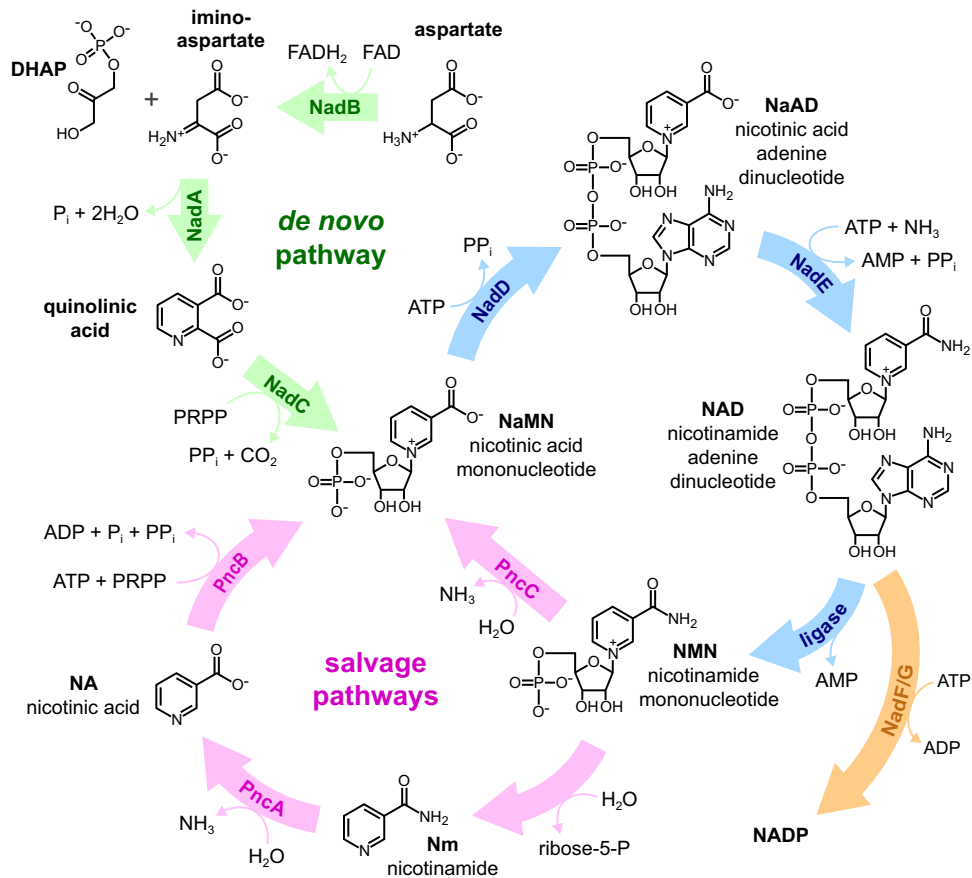


FIG 1 NAD in *Salmonella* is either synthesized *de novo* starting from aspartate and dihydroxyacetone phosphate (DHAP) or generated from Nm, NA, or quinolinic acid scavenged from outside the cell. Nicotinamide ribonucleoside (not shown) can also be taken up and converted into NMN (41). DNA ligase and other enzymes utilize NAD and break it down primarily into NMN. PRPP, phosphoribosyl pyrophosphate.

of the *pncA* locus are resistant to growth inhibition by 6ANm, while null alleles of *pncB* are resistant to both 6ANm and 6ANA. Rare mutations in the *nadB* and *nadD* genes have also been identified (6) that are resistant to both 6-amino analogs (6A^r). The 6A^r mutations in *nadB*, which encodes the first enzyme in the *de novo* pathway, are thought to make that enzyme insensitive to feedback inhibition by NAD. This results in *de novo* NAD synthesis in the presence of 6-amino-NAD. In contrast, the *nadD* mutations are believed to reduce the activity of the NadD enzyme in order to lower 6-amino-NAD levels. NadB would no longer be feedback inhibited and could begin *de novo* NAD synthesis. Unmodified NaMN may then outcompete 6-amino-NaMN for processing by the reduced-activity NadD enzyme (6, 7).

Mutants with unusual phenotypes can be distinguished from the more common loss of function mutants by screening for temperature sensitivity. A temperature-sensitive (TS) enzyme typically retains some function at a lower temperature but unfolds at higher temperatures. In addition, the 6A^r-TS alleles are readily distinguished from each other. The 6A^r-TS *pncA* and *pncB* alleles exhibit a TS phenotype only on analog-containing media since the *de novo* pathway is unaffected. Strains with the 6A^r-TS *nadB* alleles affecting *de novo* synthesis are unable to grow on minimal media at 42°C unless supplemented with Nm or NA. Strains with the 6A^r-TS *nadD* alleles are unable to grow at 42°C even in the presence of Nm or NA since the *de novo* and salvage pathways both produce NaMN substrate for the NadD enzyme (6).

In the isolation and characterization of the 6A^r-TS *nadD* alleles, we were surprised that 6A^r-TS *nadE* alleles were not isolated (6–8). Mutants with reduced-activity NadE were expected to have a phenotype similar to the *nadD* mutants. Also, all 6A^r-TS *nadD*

alleles (6) were rescued for growth at 42°C on LB medium even though they were defective in an essential gene. In an attempt to isolate either 6A^r-TS *nadE* alleles or *nadD* alleles that were TS on LB medium, an exhaustive screen for these mutant classes was performed.

RESULTS

The isolation of rare 6ANm^r temperature-sensitive mutants. For each of 200 independent cultures of wild-type *Salmonella enterica* serovar Typhimurium grown in minimal glucose medium, a 0.1-ml portion was plated on minimal glucose medium supplemented with 6ANm. After 2 days of incubation at 30°C, an average of ~250 colonies appeared per plate. These were replica printed to two minimal glucose plates supplemented with 6ANm; one was incubated at 30°C and the other was incubated at 42°C. Colonies that failed to grow at 42°C were successively single colony isolated on nutrient plates at 30°C. Up to four colonies per culture that retained a 6ANm TS phenotype were screened for defects in *pncA* and/or *pncB* (TS on minimal glucose-6ANm plates only), *nadB* (TS on minimal glucose but not on minimal glucose-NA), or *nadD* (TS on minimal glucose and minimal glucose-NA). There were 34 independent mutants isolated in the *pncA* and/or *pncB* class, 64 in the *nadB* class, and 36 in the *nadD* class from the roughly 4×10^{10} cells in the screen in total. Of the *nadD* class mutants, 34 out of 36 were found to be linked to the *nadD* locus using the *zbe-1023::Tn10* that is 50% cotransducible with *nadD* (7). One of the unlinked mutants was resistant to both 6ANm and 6ANA (similar to all characterized 6ANm^r-TS *nadD* alleles), and the other was resistant to 6ANm but not 6ANA.

As in previous screens, all 34 of the mutants with *nadD*-linked 6ANm^r-TS alleles were rescued for growth at 42°C on LB medium. Alternate pyridine sources that are not processed by NadD could explain the growth on LB medium. We tested mutants with the *nadD* 6ANm^r-TS alleles for growth on minimal glucose supplemented with NaAD or NAD at 42°C and none grew. This was expected since there is no known import mechanism for these diphosphorylated nucleotides. To verify that *nadD* is an essential gene and not being bypassed on LB medium, we knocked out *nadD* by λ -red recombination. The tetracycline resistant (Tc^r) *tetR-tetA* cassette (*tetRA*) was inserted into the *nadD* gene in a *Salmonella* Typhimurium strain containing the *Escherichia coli* F'152-2 plasmid, which includes the *E. coli nadD*⁺ gene. The resulting strain grew on minimal glucose medium. When P22 transducing phage grown on the F'152-2/*nadD::tetRA* strain was used to transfer the *nadD::tetRA* to wild-type *Salmonella* Typhimurium, colonies on LB plus Tc were obtained only if the strain carried the F'152-2(*nadD*⁺) plasmid. These results indicate that *nadD* was an essential gene and that LB medium did not contain an alternative pyridine source that could bypass NadD. We presumed that the different medium/salt conditions in LB helped *nadD*(Ts) mutants to fold properly at 42°C.

The mapping and DNA sequence of a rare 6ANm^r 6ANA^r temperature-sensitive mutant. The novel 6ANm^r 6ANA^r TS allele was mapped by Tn10-mediated Hfr formation (9) to an ~650-kb region of the chromosome between the *purE* and *pyrC* loci. Since the *pncB* gene is in this region and a *pncB* mutation can confer resistance to the 6-amino analogs, linkage to *pncB* was tested. The TS phenotype showed a 50% cotransduction linkage to a *pncB::Tn10dCm* insertion (50/100), which places the TS phenotype ~9 kb from *pncB* (10). The *aspC* gene is located ~7 kb from the *pncB* locus, and a loss-of-function allele in *aspC* could result in aspartate auxotrophy. When we constructed a Δ *aspC::tetRA* mutant, it was prototrophic at 37°C and auxotrophic for aspartate when grown at 42°C. Phage P22 grown on the 6ANm^r 6ANA^r TS allele was used to transduce the Δ *aspC::tetRA* mutant to tetracycline sensitivity (Tc^s). All Tc^s transductants (9/9) acquired the TS aspartate auxotrophic phenotype, but only a fraction of the Tc^s transductants (3/9) acquired the 6ANA^r phenotype. This suggested two separate mutations, one in *pncB* for resistance to the 6ANm and 6ANA analogs and a second mutation in *aspC* that resulted in auxotrophy for aspartate at 42°C. DNA sequence analysis revealed that the original mutant had a single-base deletion in *pncB*

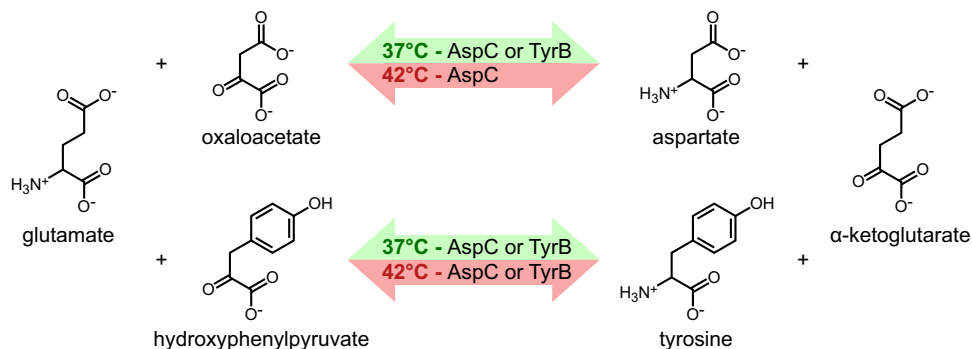


FIG 2 The aminotransferase reactions catalyzed by aspartate aminotransferase (AspC) and tyrosine aminotransferase (TyrB) have overlapping specificities at 37°C, but at 42°C only AspC is able to perform both enzymatic reactions.

(Δ62A), was deleted from bp –5 through 1002 of *aspC*, and had a single-base insertion in *aspC* (an A after bp 1075). Therefore, an unusual mutational event (or events) generated at least three mutations in the *aspC* and *pncB* region.

We were surprised to find that deletion of most of the *aspC* gene for aspartate aminotransferase resulted in aspartate auxotrophy at 42°C but not at 37°C. In *E. coli*, an *aspC* null strain grows at 37°C without added aspartate by the action of tyrosine aminotransferase, which is encoded by the *tyrB* gene (11). The AspC and TyrB aminotransferases catalyze the final step in the biosynthesis of aspartate and tyrosine by transferring an amino group from glutamate to the α-keto precursors. The effect of the *aspC* null mutation on the growth of *E. coli* in minimal medium without aspartate at 42°C had not been reported. For *Salmonella* Typhimurium at 37°C and 42°C, we found that a *tyrB* deletion was not auxotrophic for tyrosine unless *aspC* was also mutated. The *aspC tyrB* double mutant was auxotrophic for both aspartate and tyrosine, and the *aspC* single mutant was auxotrophic for aspartate only at 42°C. These results, diagrammed in Fig. 2, suggest that both AspC and TyrB can produce aspartate and tyrosine but that TyrB cannot synthesize aspartate at 42°C.

We attempted to isolate mutants in *tyrB* by spreading 0.1 ml of overnight cultures of the Δ*aspC* strain on minimal glucose plates at 42°C. Revertants were found to have mutations linked to the *tyrB* locus. DNA sequence analysis of four independent revertants revealed that two contained base substitutions in the *tyrB* promoter region. Both base substitutions mutated the putative σ⁷⁰ promoter toward the canonical sequence (TCGATC-N₁₇-TATTGT to either TTGATC-N₁₇-TATTGT or TCGATC-N₁₇-TATTAT; the canonical σ⁷⁰ promoter sequence is TTGACA-N₁₇-TATAAT). A third mutation 21 bp upstream of *tyrB* (G-21A) was in the putative untranslated region of the mRNA. A fourth mutation resulted in a synonymous GAC to GAT aspartate codon change for amino acid 6 of TyrB. It is likely that this synonymous change results in increased *tyrB* mRNA translation. A similar synonymous D12D change in the *fliA* gene resulted in an ~2.5-fold increase in *fliA* mRNA translation in *Salmonella* Typhimurium (12, 13).

The mapping of a rare 6ANm^r 6ANA^s temperature-sensitive mutant. The second TS mutant was 6ANm^r and 6ANA^s, indicating a defect in the nicotinamide deamidase (PncA) step of the NAD metabolic pathway. However, no mutant had ever been isolated with a 6ANm^r 6ANA^s phenotype that could not grow on minimal glucose plates (with or without added NA). The TS mutation was mapped by Tn10-mediated Hfr formation (9) to an ~550-kb region of the chromosome between the *pyrC* and *pyrF* loci, which includes *pncA*. Tn10 insertions linked to the TS mutation were isolated. Unlike the 6ANm^r 6ANA^r TS mutant discussed earlier, the TS phenotype and 6ANm^r phenotype were 100% linked to each other. In addition, when phage P22 grown on a strain containing both the TS allele and a linked Tn10 insertion was transduced into a wild-type strain (selecting for transfer of the Tc^r Tn10 insertion), none coinherited the TS allele (<1%). This is the expected linkage phenotype of a large DNA insertion

TABLE 1 The TS insertion allele reduces *pncA* transcription and increases expression of the SOS-inducible genes *mucB* and *umuC*

LacZ reporter	β -Gal activity in ^a :		
	Wild type	TS insertion mutant	TS insertion/wild type ratio
<i>pncA247::MudA</i>	13	1.7	0.13
<i>pncA249::MudA</i>	28	5.9	0.21
<i>pncA251::MudA</i>	20	3.2	0.16
<i>pncA252::MudA</i>	27	4.3	0.16
<i>pncA212::MudA</i>	29	5.8	0.20
<i>pncA229::MudA</i>	30	5.5	0.18
pSE200 (<i>mucB::lacZ</i>)	8.7	46	5.3
pSE143 (<i>umuC::lacZ</i>)	2.9	18	6.2

^aAll β -galactosidase activities represent the averages from triplicate assays with a deviation of less than $\pm 20\%$ and are expressed in Miller units (38).

mutation that can be repaired in the recipient but not moved as a donor allele by P22-mediated transduction. Thus, the above results are consistent with the 6ANm^r and TS phenotypes being caused by a single large DNA insertion.

Mud-*lac* transcriptional fusions to *pncA* revealed 5- to 7-fold lower transcription of *pncA* in the presence of the TS allele (Table 1). The *pncA* gene is transcribed in two operons in *Salmonella* Typhimurium. The *sppA-ansA-pncA* operon accounts for $\sim 90\%$ of nicotinamide deamidase activity in the cell, while the remaining $\sim 10\%$ is expressed from the *ansA-pncA* transcript (14). An insertion in *sppA* that reduced *pncA* transcription would explain the 6ANm^r and 6ANA^s phenotype but not the TS growth defect. Neither the signal peptide peptidase gene *sppA* nor the L-asparagine synthetase gene *ansA* is essential; oligopeptidase A also cleaves signal peptides, and a second L-asparagine synthetase is encoded by the *asnB* locus (15, 16).

The insertion mutant is induced for the SOS DNA repair response. The cells of the *sppA-ansA-pncA* insertion mutant are typically twice as long as the wild type, and there are many more filamentous cells than in a wild-type culture. A filamentous phenotype is characteristic of the SOS repair response associated with DNA damage (17). Two SOS reporter plasmids were introduced into the mutant and wild-type strains that carried *lacZ* gene fusions to either the *E. coli* *mucB* or *umuC* SOS-inducible genes (17). In the presence of the *sppA-ansA-pncA* insertion, β -galactosidase (β -gal) levels were up 5- to 6-fold compared to the parent strain (Table 1). These data suggest that the SOS DNA repair response was induced in the insertion mutant, but it was not clear why.

The large insertion could be generating DNA damage by reducing expression of nearby DNA metabolism genes. This insertion is near two genes involved in the repair or protection of DNA. The first is ~ 9 kb away and encodes the structural gene for exonuclease III (Exo III) (*xthA*) (18). Exo III has endonuclease and exonuclease activities and repairs apurinic and apyridinic sites in DNA (18, 19). After Exo III removes DNA damage, DNA polymerase I and DNA ligase repair the single-stranded DNA gaps (20). The Exo III levels from crude cell extracts showed a 40-fold increase over wild-type cell extracts (Table 2). Exo III levels were also determined for 10 independent spontaneous revertants that grew on minimal glucose medium at 42°C. All 10 showed Exo III levels similar to those of the wild type. We surmised that single-stranded DNA could accumulate if Exo III hyperactivity outcompeted PolI for closing gaps and that these single-stranded regions could then induce the SOS response.

The other DNA metabolism gene linked to *pncA* (*katE*) is ~ 28 kb away and encodes catalase. Catalase removes hydrogen peroxide (H₂O₂) through enzymatic conversion to O₂ and H₂O. Peroxide is known to damage DNA, and the SOS response increases resistance to killing by peroxide (21). We tested the *sppA-ansA-pncA* insertion mutant and the 10 revertants that were able to grow at 42°C for sensitivity to killing by hydrogen peroxide added to the growth medium (Table 2). While the *sppA-ansA-pncA* insertion mutant did exhibit resistance to peroxide killing as expected, two of the ten

TABLE 2 Exonuclease III activity in crude cell extracts and resistance to hydrogen peroxide of the *sppA-ansA-pncA* insertion mutant and 42°C⁺ revertants

Strain	Genotype	Exonuclease activity ^a	% survival after H ₂ O ₂ treatment ^b
LT2	Wild type	6.2	7.6
TR6630	<i>sppA-ansA-pncA</i> insertion	245	26
TR6632	42°C ⁺ revertant of TR6630	5.9	4.0
TR6633	42°C ⁺ revertant of TR6630	5.6	7.7
TR6634	42°C ⁺ revertant of TR6630	5.4	5.4
TR6635	42°C ⁺ revertant of TR6630	4.7	7.1
TR6636	42°C ⁺ revertant of TR6630	4.8	19
TR6637	42°C ⁺ revertant of TR6630	5.8	29
TR6638	42°C ⁺ revertant of TR6630	6.8	4.1
TR6639	42°C ⁺ revertant of TR6630	3.8	0.2
TR6640	42°C ⁺ revertant of TR6630	5.4	7.7
TR6641	42°C ⁺ revertant of TR6630	5.5	8.5

^aActivity is expressed in micrograms of DNA degraded per minute per milligram of protein.

^bThe percentage compares the number of viable colonies from cells exposed to 5 mM H₂O₂ for 30 min to the number of colonies from untreated cells.

42°C⁺ revertants were also resistant to peroxide killing, and one revertant (TR6639) was hypersensitive to peroxide killing.

Genome analysis of the insertion mutant. The nature of the *sppA-ansA-pncA* insertion mutant and the cause of the temperature-sensitive phenotype remained elusive until the entire genome was sequenced. Genome sequencing revealed that the 94-kb virulence plasmid pSLT had inserted into the *sppA* gene, with 5 bp of homology at the junctions (GTAGT). This *sppA* insertion would block *pncA* transcription from the *sppA* promoter and result in the 6ANm^r 6ANA^s phenotype. In addition, the *sppA*::pSLT mutant presented an unusual pattern of read coverage. As shown in Fig. 3, the coverage of sequencing reads is uniform across the genome for the wild-type strain. In contrast, there is a peak in read coverage at the mutant's pSLT insertion site that decreases to a low point at the replication termination site. The increased sequence coverage near *sppA* likely results from DNA replication initiated from pSLT. Read coverage for the Fels-1 and Gifsy-1 prophages is also increased, which suggests that the SOS response is prompting some of the prophages to abandon ship.

Two revertants with MudJ transposon insertions in the *sppA*::pSLT background were isolated that could grow on minimal glucose plates at 42°C. These MudJ transposons restored even read coverage throughout the chromosome (Fig. 3). Genome sequence analysis revealed the MudJ transposons had inserted into the pSLT DNA and deleted 34 kb of the pSLT plasmid plus either 10 kb (to the *astA* locus) or 15 kb (to the *spy* locus) of adjacent chromosomal DNA. These deletions included the two replication origins of pSLT as well as *xthA* (Exo III) from the chromosome. The genes of the first and second pSLT replication origins are homologous to the R100 and phage P1 plasmid origins, respectively.

A series of deletions were constructed by λ -red recombination to determine the cause of temperature sensitivity (Fig. 4). Deleting *xthA* (Exo III) did not restore growth at 42°C, but deletions encompassing both pSLT replication origins did allow wild-type growth ($\Delta traH$ -PSLT026 and $\Delta repC$ -*repA2* mutants). Deleting the first replication origin slightly increased the growth rate ($\Delta repC$ -*repA* mutant), and deleting the second replication origin allowed 1 in 1,200 cells to produce normal-sized colonies ($\Delta repA2$ mutant). Wild-type growth at 42°C was restored only when both replication origins were deleted ($\Delta repC$ -*repA* $\Delta repA2$ double mutant). These data suggest that replication initiated from the integrated pSLT plasmid is responsible for the SOS response and growth defect.

DISCUSSION

The extensive screen for *nad* mutants in this study identified two rare temperature-sensitive mutations outside of the NAD pathway. The first strain contained mutations

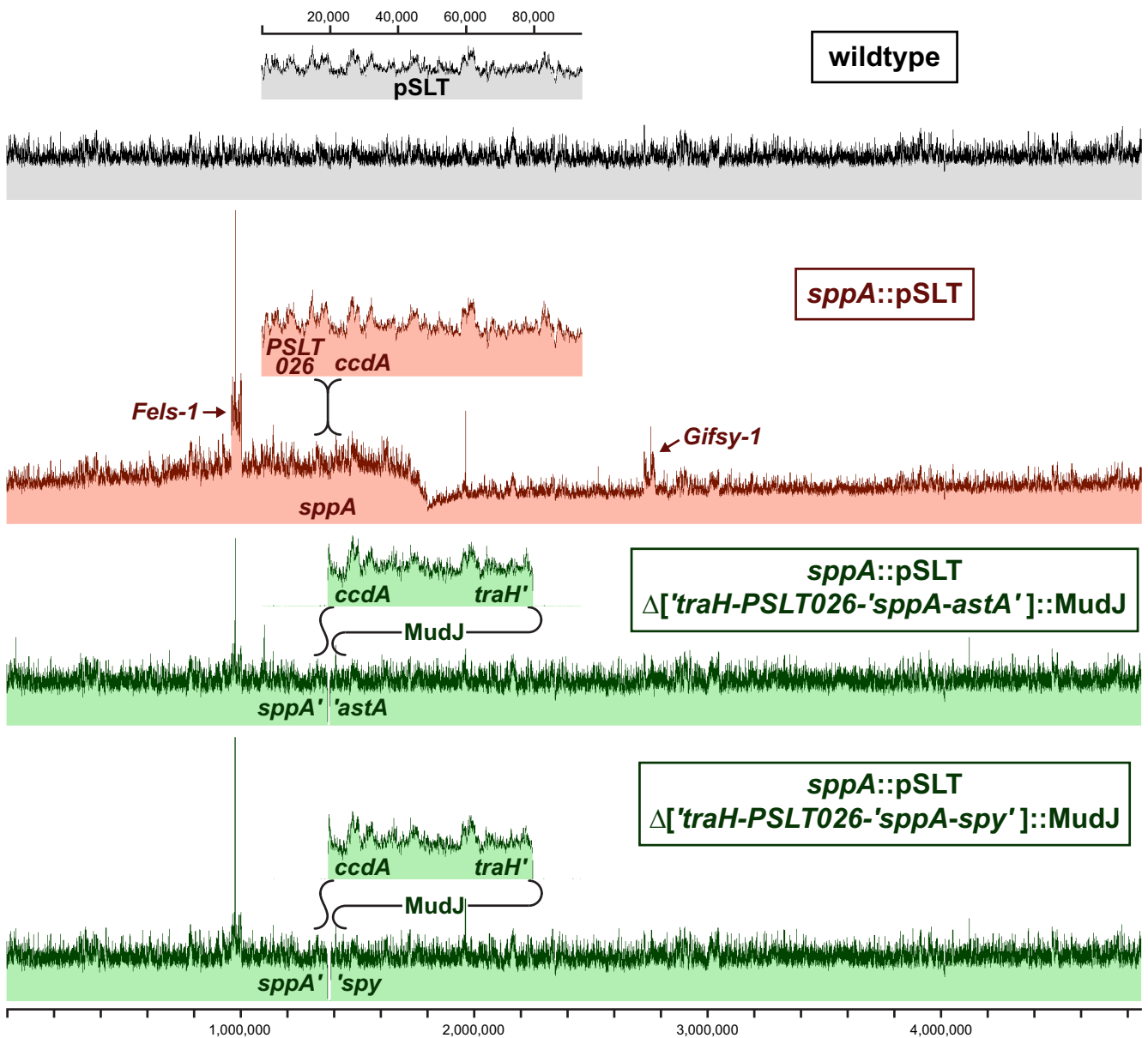


FIG 3 Insertion of the virulence plasmid into the chromosome distorts read coverage from Illumina sequencing of genomic DNA. The coverage is scaled so that the average chromosomal coverage is the same between the strains. The dark top edge extends from the maximum coverage to the average coverage for the sequence represented by each pixel. Genomic DNA was harvested from stationary-phase cells after growth in LB at 37°C. The 11-kb MudJ transposons are inserted at two different positions within *traH* and delete 34 kb of pSLT plus 10 kb or 15 kb of adjacent chromosomal DNA.

in both *pncB* and *aspC*, which are 7 kb apart. The *aspC* mutations resulted in aspartate auxotrophy only at 42°C. We found that the TyrB tyrosine aminotransferase can cover for the AspC aspartate aminotransferase but not at 42°C. There has been much interest in mutating these two aminotransferases to change their substrate specificities and even to convert them into decarboxylases or desulfonases (22–24). If TyrB has decreased activity for producing aspartate at 42°C, this observation could benefit future mutational and structural studies with these aminotransferases.

In the second temperature-sensitive mutant, the insertion of the 94-kb pSLT virulence plasmid into the *sppA* gene reduced transcription of the downstream *pncA* gene. The temperature sensitivity resulted from pSLT replication of the chromosome, which triggered an SOS response. Extra replication origins are known to cause problems for cells. Replication proceeding in the wrong direction through the highly transcribed rRNA operons causes collisions between the replication forks and RNA polymerase (25,

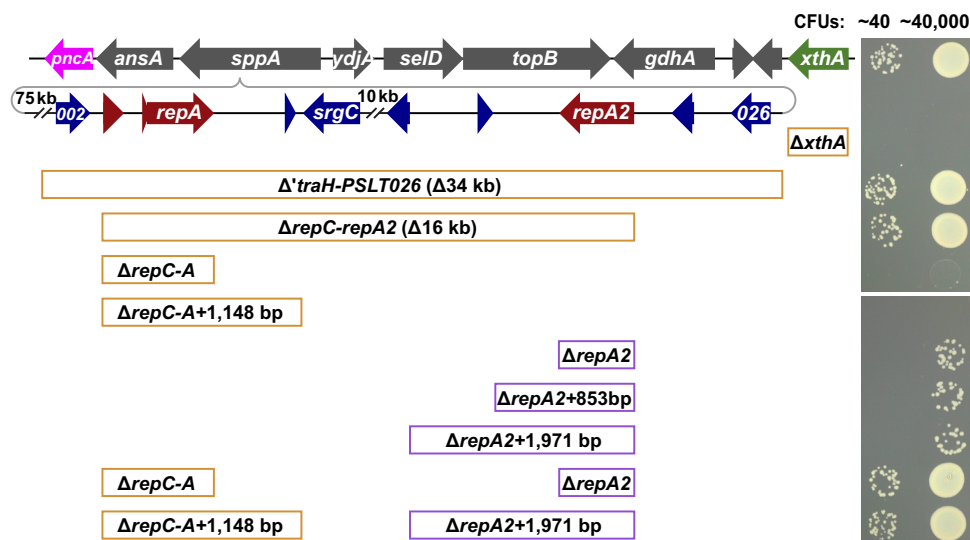


FIG 4 Deletion of both pSLT replication origins is required for growth of the *sppA*::pSLT mutant on minimal glucose plates at 42°C. LB overnight cultures were diluted in buffered saline and spotted onto minimal glucose plates for 40 or 40,000 CFU per spot. The plates were incubated at 42°C for 24 h. The top line represents the wild-type *Salmonella* Typhimurium strain, and the second line represents the *sppA*::pSLT insertion mutant. The remaining strains include one or two deletions in the *sppA*::pSLT background. Each deletion inserted either a 1-kb *Cm^r* cassette (orange boxes) or a 1.5-kb *Kan^r* cassette (purple boxes) into the deleted region. The upper and lower images are from different parts of the same plate. Red arrows indicate replication genes. Dilutions were also spotted onto LB plates and grown at 30°C for colony counts (not shown).

26). In addition, cells do not survive too much replication initiation, even from the normal replication initiation region of the chromosome (27). In the case of the pSLT insertion, replication forks would advance the wrong way through five of the seven rRNA operons. If the pSLT origins are more active on minimal medium at 42°C, the temperature sensitivity of this mutant strain could be explained.

Insertions of pSLT could be useful for probing genome structure. If one or both pSLT replication origins were cloned into a transposon, pSLT origins could be inserted randomly into a target chromosome. Sequence coverage from next-generation sequencing (NGS) could then quickly determine whether replication is terminated in a specific region or simply halfway around the chromosome where the replication forks meet. In addition, these transposon insertions could expose large chromosomal rearrangements that disrupt the gradient of sequence coverage. For example, a large inversion would reverse the sequence gradient and introduce jumps in coverage at the junctions. NGS of genomic DNA extracted from exponentially growing cultures is another method for generating a sequence coverage gradient to detect rearrangements (28). However, this other method has difficulty detecting rearrangements that swap DNA between the left and right arms of the chromosome without changing their orientation to the origin. Because highly expressed genes would continue to transcribe in the same direction as replication, these rearrangements may be more stable and more common (29). A pSLT replication origin inserted away from the chromosomal replication origin would allow these rearrangements to be identified. This technique could be particularly useful the first time a species is sequenced to verify that the genome was properly assembled.

MATERIALS AND METHODS

Bacterial strains and growth media. Strains used in this study are listed in Tables 2 and 3. All *Salmonella enterica* serovar Typhimurium strains were derived from wild-type strain LT2. The E medium of Vogel and Bonner (30), supplemented with 0.2% D-glucose, was used as minimal medium. Either LB medium or Difco nutrient broth (8 g/liter) was used as rich medium for growth of bacterial cultures. Solid medium was supplemented with either 15 g/liter Difco agar or 12 g/liter Apex chemical reagent agar. Auxotrophic supplements were included in media as recommended by Davis et al. (31). Antibiotics were added to media as follows (final concentrations given): tetracycline hydrochloride (15 μg/ml in rich

TABLE 3 List of strains

Strain	Genotype	Source or reference ^a
TH437	Wild-type strain LT2	Lab collection
TH1184	F'152-2/ <i>nadA56 nadD364</i> ::MudJ	
TH1898	<i>pncB</i> ::Tn10dCm	
TH2117	<i>sppA</i> ::pSLT::MudJ (Δ 975 bp into <i>traH</i> through 676 bp into <i>astA</i>)	
TH2119	<i>sppA</i> ::pSLT::MudJ (Δ 1,019 bp into <i>traH</i> through 438 bp into <i>spy</i>)	
TH4875	<i>zbe-1023</i> ::Tn10 (near <i>nadD</i>)	7
TH22933	Δ <i>tyrB946</i> ::FKF (retains first five and last five amino acids)	
TH23051	Δ <i>aspC881</i> :: <i>tetRA</i> (retains first five and last five amino acids)	
TH23053	Δ <i>aspC882</i> (clean deletion; retains first five and last five amino acids)	
TH23095	Δ <i>tyrB946</i> ::FKF Δ <i>aspC882</i>	
TH23393	Δ <i>aspC881</i> :: <i>tetRA tyrB949</i> (C18T)	
TH23394	Δ <i>aspC881</i> :: <i>tetRA P_{tyrB}950</i> (G-40A)	
TH23395	Δ <i>aspC881</i> :: <i>tetRA P_{tyrB}951</i> (C-66T)	
TH23396	Δ <i>aspC881</i> :: <i>tetRA P_{tyrB}952</i> (G-21A)	
TH23806	F'152-2/ <i>nadA56 nadD956</i> :: <i>tetRA</i> (inserted after amino acid 105)	
TR6630	<i>sppA</i> ::pSLT (156 bp before <i>PSLT026</i> inserted 1,055 bp into <i>sppA</i>)	
TR6631	<i>pncB255</i> (Δ 62A) <i>aspC883</i> (Δ -5 to 1,002 bp; A inserted after 1,075 bp)	
TT8361	pSE200 (<i>mucB</i> :: <i>lacZ</i>)/AB1157 <i>thy lexB30</i>	42
TT8362	pSE143 (<i>umuC</i> :: <i>lacZ</i>)/AB1157 <i>thy lexB30</i>	42
TT9993	<i>sppA</i> ::pSLT <i>pncA247</i> ::MudA	
TT9994	<i>pncA247</i> ::MudA	
TT9997	<i>sppA</i> ::pSLT <i>pncA249</i> ::MudA	
TT9998	<i>pncA249</i> ::MudA	
TT10001	<i>sppA</i> ::pSLT <i>pncA251</i> ::MudA	
TT10002	<i>pncA251</i> ::MudA	
TT10003	<i>sppA</i> ::pSLT <i>pncA252</i> ::MudA	
TT10004	<i>pncA252</i> ::MudA	
TT10005	<i>sppA</i> ::pSLT <i>pncA212</i> ::MudA	
TT10006	<i>pncA212</i> ::MudA	
TT10009	<i>sppA</i> ::pSLT <i>pncA229</i> ::MudA	
TT10010	<i>pncA229</i> ::MudA	
TT10024	pSE200(<i>mucB</i> :: <i>lacZ</i>)	
TT10025	pSE143(<i>umuC</i> :: <i>lacZ</i>)	
TT10026	pSE200(<i>mucB</i> :: <i>lacZ</i>)/ <i>sppA</i> ::pSLT	
TT10027	pSE143(<i>umuC</i> :: <i>lacZ</i>)/ <i>sppA</i> ::pSLT	
TT10044	<i>pnc-254</i> ::Tn10 (near <i>sppA</i>)	
TT10281	<i>zcg-1819</i> ::Tn10 (linked to <i>pncA</i>)	
TGL553	<i>sppA</i> ::pSLT(Δ <i>repC-repA</i> ::FCF) (Δ <i>repC</i> to 1 bp after stop of <i>repA</i>)	
TGL555	<i>sppA</i> ::pSLT(Δ <i>repC-repA2</i> ::FCF) (Δ <i>repC</i> through -1 of <i>repA2</i>)	
TGL556	<i>sppA</i> ::pSLT(Δ <i>traH-PSLT026</i> ::FCF) (Δ 975 bp of <i>traH</i> to pSLT end)	
TGL557	<i>sppA</i> ::pSLT Δ <i>xthA</i> ::FCF (deleted all but last bp of <i>xthA</i>)	
TGL584	<i>sppA</i> ::pSLT(Δ <i>repA2-1</i> ::FKF) (deleted -1 through stop)	
TGL585	<i>sppA</i> ::pSLT(Δ <i>repA2-2</i> ::FKF) (deleted -1 through 855 bp after <i>repA2</i>)	
TGL586	<i>sppA</i> ::pSLT(Δ <i>repA2-pefB</i> ::FKF) (deleted -1 to 1,974 bp after <i>repA2</i>)	
TGL587	<i>sppA</i> ::pSLT(Δ <i>repCA-PSLT007</i> ::FCF) (Δ <i>repC</i> to 1,150 bp after <i>repA</i>)	
TGL596	<i>sppA</i> ::pSLT(Δ <i>repC-repA</i> ::FCF Δ <i>repA2-1</i> ::FKF)	
TGL601	<i>sppA</i> ::pSLT(Δ <i>repCA-PSLT007</i> ::FCF Δ <i>repA2-pefB</i> ::FKF)	

^aUnless noted otherwise, all strains were constructed during the course of this work.

medium and 7.5 μ g/ml in minimal medium), kanamycin sulfate (50 μ g/ml in rich medium and minimal medium), chloramphenicol (12.5 μ g/ml in rich medium and 6.25 μ g/ml in minimal medium), streptomycin sulfate (1 mg/ml), and sodium ampicillin (100 μ g/ml for multicopy plasmid selection and 30 μ g/ml or 15 μ g/ml for single copy selection of chromosomal MudA insertions in LB or minimal medium, respectively). Pyridine nucleotides were added as follows (final concentrations given): nicotinic acid and nicotinamide (2 μ g/ml), 6-aminonicotinic acid and 6-aminonicotinamide (50 μ g/ml), NAD and NaAD (0.5 mM). X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) dissolved in *N,N*-dimethylformamide (20 mg/ml; Fisher Scientific Co.) was added to medium at a final concentration of 25 μ g/ml.

DNA sequencing and λ -red recombination. Primers are listed in Table 4. PCR products were Sanger sequenced at either Genewiz (South Plainfield, NJ) or Eton Bioscience (San Diego, CA). DNA primers were synthesized at Eton Bioscience. Chromosomal Tn10 insertion sites were determined by arbitrary PCR and Sanger sequencing (32). The FRT-Cm-FRT cassette from pKD3 and the FRT-Kan-FRT cassette from pKD4 (33) and the *tetRA* cassette from Tn10 were amplified using primers that added about 40 bp of homology to each side of the cassette. Strains containing the λ -red recombination genes on pKD46 (33) were grown at 30°C in 25 ml of LB-Amp-Ara to an optical density at 600 nm (OD₆₀₀) of 0.6, washed twice in 25 ml of cold water, and resuspended in 100 μ l of cold water, and a 50- μ l portion was mixed with PCR product. After electroporation and 1 h outgrowth, recombinants were selected on plates containing the appropriate antibiotic.

TABLE 4 List of primers

Primer name	Sequence
7319 - tyrB FRT fwd	CTCTGTAACCTGGAGAACCATCGCGTGTTCAAAAAGTTGTGTAGGCTGGAGCTGCTTC
7320 - tyrB FRT rev	TTATCGCCGTCCGGCCTGAATATTACATGACAGCGGCAACATATGAATATCCTCCTTAG
7345 - DaspC-tetR	ATCGGTACGCCAGTCGGCAGCTTTTTACAGTACCGCGACTTAAGACCCACTTTTACATT
7346 - DaspC-tetA	TCTGTAACATAATGGAACCTCGTCATGTTTGAGAACAATAAGCACTTGTCTCCTG
7356 - DaspC-clean	ATCGGTACGCCAGTCGGCAGCTTTTTACAGTACCGCGACTATGTTCTCAAACATGACGAGGTTCCATTATAGTTACAGA
7357 - DaspC-fillin	TCTGTAACATAATGGAA
7760 - nadD-aa108-tetR	GCGCGAAGAACAGGGCCCCGAAGCGCCGCTGGCGTTATTTTAAAGACCCACTTTTACATT
7761 - nadD-aa108-tetA	CATGCCAGGTGGGGAAGTTAAGCAGCGAGTCTCGGCCGATCTAAGCACTTGTCTCCTG
8181 - traHp2	GATTACGAAGCTGCTGACCAGTATTCAGAACAAGGCGGTATGAATATCCTCCTTAG
8182 - sppAp1	TGGCGAAGAAACGCGAGGGAATGTCGGCGCGGACACTACGTGTAGGCTGGAGCTGCTTC
8183 - repCp2	TTTTAGAAATCTGTAGTATTCTCTGCAAACGATCCAGGTATGAATATCCTCCTTAG
8184 - repAp1	AAATCCTCCGAGCTAGCCGGATTTTATAATCTGGTGGGTGTAGGCTGGAGCTGCTTC
8185 - repA2p2	ATAAGTAAACAGTAATGGATAACTACTACAGATTATGTCATATGAATATCCTCCTTAG
8186 - repA2p1	CGGAGGCGCTCTGGACAAGGACAATCTGGACATAAAAAAGTGTAGGCTGGAGCTGCTTC
8187 - xthAp2	CGCGCTATCCCGGCTTACGGTAAATCAGAAAGCGGAATCATATGAATATCCTCCTTAG
8188 - xthAp1	CCATCCCAACACGATTACCTTGCAAAGTGGCAGTGACTGTGTAGGCTGGAGCTGCTTC
8320 - repAoriP1	TATTGCAAAATAACAGTAAACCGTGTATAACATCCGGCTGTAGGCTGGAGCTGCTTC
8321 - repA2oriP2	AAAACATCCAAGTGTGAAGTGATACCGGAGTTTAAAGAACATATGAATATCCTCCTTAG
8322 - repA2pefP2	TTGCCCAAATAAATCAGCATCTTTTCTGTTACAGCATCATATGAATATCCTCCTTAG

Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue kit from stationary-phase cultures grown overnight in LB at 37°C. The High-Throughput Genomics Shared Resource at the Huntsman Cancer Institute then prepared the DNA with the Illumina TruSeq DNA PCR-free kit and sequenced 50-nucleotide (nt) single reads on an Illumina HiSeq 2500 instrument. The sequencing reads were aligned to the *Salmonella* Typhimurium, pSLT, and PhiX174 genomes using the Geneious “map to reference” tool with the Geneious mapping algorithm. Homology was determined using Pfam (34), InterPro (35), and BLAST (36).

Genetic manipulations, enzyme assays, and other techniques. To isolate spontaneous revertants of TS mutants, 0.1 ml of an LB overnight culture was spread onto a minimal glucose plate and incubated at 42°C. For the MudJ revertants, the MudJ was transposed as described (37) into the *sppA::pSLT* strain. Colonies that grew on LB-kanamycin plates were replica printed to minimal glucose plates and incubated at 42°C. Transduction and conjugation methods were performed as described (7). β -Galactosidase was assayed as described by Miller (38) using the CHCl_3 -sodium dodecyl sulfate permeabilization procedure. Exonuclease III assays were performed according to Rogers and Weiss (39). Cell lengths were observed at $\times 400$ magnification using phase contrast on a Zeiss PrimoStar microscope.

Sensitivity to hydrogen peroxide was tested as described for *E. coli* (40) with minor modifications. Cultures of bacteria were grown overnight at 37°C in Difco nutrient broth. Overnight cultures were diluted 100-fold into minimal medium and grown to mid-log phase (OD_{600} of ~ 0.6). A 16- μl portion of a freshly prepared H_2O_2 solution was added to 3 ml of culture for a final concentration of 5 mM H_2O_2 . The cultures were shaken at 30°C for 30 min and then diluted and spread on plates to determine the number of viable colonies compared to the pretreated culture.

Data availability. The strains and plasmids are available upon request and should be sufficient to verify the results and conclusions of this study. The next-generation sequencing read data have been deposited in the Sequence Read Archive under accession numbers [SRR12129225](https://www.ncbi.nlm.nih.gov/sra/SRR12129225), [SRR12129226](https://www.ncbi.nlm.nih.gov/sra/SRR12129226), and [SRR12129227](https://www.ncbi.nlm.nih.gov/sra/SRR12129227).

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