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NOTE

Identification of Genes That Are Dispensable for Animal Infection by *Salmonella typhimurium*

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In the current study, we generated a pool of *Salmonella typhimurium* mutants using the Tn*10*d-cam mini-transposon. This pool of mutants was administered to BALB/c mice through the oral route, and bacteria were recovered from the spleen 3 days post-infection. After three rounds of serial passage, we observed enrichment of two insertion mutants, a *yddG* insertion and an *amyA* insertion. These two genes have been implicated in growth on plant products (*amyA*) and survival in the presence of paraquat (*yddG*), both of which are natural environments for *Salmonella*. Thus, while *in vivo* expression technology has identified *S. typhimurium* genes that are absolutely necessary for animal infection, other genes involved in vegetative growth also appear to play role in the establishment of pathogenesis.

***Keywords*:** *Salmonella*, transposon mutagenesis, *amyA*, *yddG*, pathogenic genes

Salmonella enterica serovar typhimurium, a member of Enterobacteriacease, is a ubiquitous microorganism found in soil, water, and vegetation, as well as animals, where it causes enteritis, septicemia and enteric fever. In mice, *Salmonella typhimurium* causes a systemic, typhoid-like disease (Cohen *et al.*, 1987). Thus, *Salmonella* are equipped to survive in the natural environment and are also able to infect suitable animal hosts. Following ingestion by a susceptible animal, *S. typhimurium* colonizes the intestinal tract, penetrates the intestinal epithelium and gains access to systemic sites such as the spleen and liver through lymphatic and blood circulation (Carter and Collins, 1974). It has been reported that approximately 4% of the *S. typhimurium* genome, encompassing approximately 200 virulence genes, is required for fatal infection in mice (Bowe *et al.*, 1998; McClelland *et al.*, 2001). Many of these genes are clustered on *Salmonella* pathogenicity islands (SPIs), which are composed of a series of genes and operons and play a crucial role in the pathogenesis of *S. enterica* (Marcus *et al.*, 2000). Among the five SPIs identified to date, SPI-1 has been shown to play an important role in invasion of epithelial cells (Galan and Curtiss, 1989), whereas SPI-2 is required for bacterial replication within macrophages (Ochman *et al.*, 1996) and systemic growth in the mouse (Hensel *et al.*, 1997). In addition to SPI-encoded virulence genes, several other genes have been shown to be necessary for *S. typhimurium* pathogenicity (Groisman and Ochman, 1997), many of which are involved in intracellular survival and replication. These include the *spv* locus on a resident plasmid (Gulig *et al.*, 1993), the *ompR*/*envZ* genes, which encode a two-component regulatory system that responds to changes in pH, osmolarity, and temperature (Heyde and Portalier, 1987; Thomas and Booth, 1992), and the *phoP*/*phoQ* genes, which encode another two-component regulatory system that controls the expression of approximately 40 virulence genes (Miller and Mekalanos, 1990).

Genes implicated in *Salmonella* virulence have been identified in an attempt to understand the mechanism of bacterial pathogenesis. Several *in vivo* experimental systems, referred to as *in vivo* expression technology (IVET), for identifying virulence genes have been described. One such system, signature-tagged mutagenesis (STM), relies on comparative hybridization to identify transposon insertion mutants that are unable to survive in a particular host (Andrews-Polymenis *et al.*, 2009). Recently, using STM, 189 bacterial genes were identified in bacteria isolated from the spleens of BALB/cJ mice infected with a library of *Salmonella* transposon insertion mutants (Chan *et al.*, 2005). Of the isolated mutants that were unable to survive in mice, a significant proportion carried mutations in either components of SPI2 or factors involved in lipopolysaccharide synthesis. Although this type of negative selection also identified other genes with putative functions, it was generally assumed that most genes, including biosynthesis genes needed for vegetative growth in natural environments, would be dispensable for bacterial survival in mice.

In the current study, we generated a pool of mutagenized *S. typhimurium* using the mini-transposon Tn*10*d-cam and isolated mutants that survived best in mice. Interestingly, we observed an enrichment of only two types of transposon insertion mutants, those that carried insertions in *yddG* and *amyA*, both of which are implicated in growth only in natural environments.

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Bacterial strains and genetic manipulation

To generate a pool of transposon mutants, *S. typhimurium* strain 14028s was transduced with P22HT *int* grown in TT10605 (LT2, *proAB-47*, F'128 pro$^+$ lac$^+$ *zzf*-1837::Tn*10*d-cam) (a generous gift from J. Roth), as described previously (Davis, 1980). Transductants were plated and chloramphenicol (CM)-resistant mutants were selected on Volgel-Bormer (VB) minimal salts medium supplemented with 0.2% glucose and CM (15 μg/ml).

Media, chemicals, and enzymes

The rich medium was Luria-Bertani (LB) broth and minimal salts VB medium supplemented with 0.2% glucose. For the solid support medium, 1.5% granulated agar (Difco Laboratories, USA) was included. Brain heart infusion media (Difco Laboratories) was used for enumeration of intracellular bacteria.

Nested PCR

For nested PCR, we used one primer of defined sequence and an ambiguous primer designed to misprime at nearby sites (Kofoid *et al.*, 1999). The sequences of the primers were as follows: NX1 (ambiguous primer), 5′-ACTTCTCAACAACTC AGGACGA ACA(N)$_{10}$ACAGC-3′; NX2 (defined sequence) 5′-ACTTCTCAACAACTCAGGACGAACA-3′; and CMR, 5′-GTCACAGGTATTTATTCGGCGCA-3′ found in CM resistant gene. Initial primer extension was carried out using NX1 and CMR at an annealing temperature of 40°C. Extension times were less than 1 min, so most of the products were short enough to be readily eluted. Wizard PCR columns (Promega, USA) were used to remove the primers and most of the large template DNA, which binds irreversibly to the columns. The eluant was then used as template in standard amplification reactions containing the CMR and NX2 primers. Reamplification with a nested primer of defined sequence was used to identify correctly anchored fragments.

Southern blot analysis

Genomic DNA was isolated using a Puregene DNA Purification kit (QIAGEN, USA). DNA samples were digested for 3 h at 37°C with *Eco*RI (New England BioLabs, USA) and then subjected to 0.7% gel electrophoresis. DNA was transferred to positively charged nylon membranes by passive transfer, as previously described (Sambrook and Russell, 2001). The membrane was exposed twice to UV light (1200 J), dried in a 64°C oven and then incubated with probe. A fragment of Tn*10*d-cam amplified by PCR was labeled using the random prime Labeling System (GE Healthcare, USA) and used as the probe.

Mixed infection of mice

Female BALB/c mice weighing 20 to 25 g were inoculated through the oral route with 0.2 ml of physiological saline containing 1×10^5 colony forming units (CFU) of bacteria. Bacteria were grown overnight at 37°C in LB medium with aeration, diluted into fresh medium (1:40) and then grown for 4 h to early stationary phase. The mixed inoculum was prepared in PBS at a concentration of 2.5×10^5 CFU bacteria/ml/strain (input). CFU of each strain in the mixed inoculum were enumerated by plating a series of dilutions of the inoculum onto LB agar and LB agar plus CM to distinguish between wild-type and Tn*10*d-cam insertion mutants. Mice were sacrificed 3 days post-infection with wild-type and Tn*10*d-cam insertion mutants. The spleens were removed, placed in sterile phosphate buffered saline (PBS), and then homogenized by mechanical disruption. After homogenization, the samples were allowed to settle on ice for 5 min before transfer of the supernatants into a fresh tube. Bacteria were pelleted by centrifugation at 15,000×g and then resuspended in sterile PBS. Bacterial CFU were enumerated by plating, as described above. The competitive index (CI) was calculated as follows: (% strain A recovered/% strain B recovered)/(% strain A inoculated/% strain B inoculated). The CI for each set of assays was analyzed statistically using the Student's t test.

Invasion assay

Invasion assays were performed essentially as described previously (Lee *et al.*, 1992). Briefly, monolayers of mouse leukemic monocyte macrophage RAW264.7 cells were prepared by seeding 5×10^5 cells onto a 24-well plate. RAW cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C with 5% CO_2. *Salmonella* prepared as described in the text were added to the RAW cells at a ratio of 10:1, and the mixture was incubated at 37°C under 5% CO_2 for 30 min. Infected cells were washed three times with PBS pH 7.4, and then DMEM containing gentamicin (5 μg/ml; Sigma, USA) was added and the cells were allowed to incubate for an additional 60 min. Intracellular bacteria were harvested by extraction with lysis buffer (0.05% Triton X-100 in PBS, pH 7.4) and then replica plated for colony counting on brain heart infusion agar plates.

Isolation of *S. typhimurium* survival mutants from mice

Our goal was to determine whether we could isolate Tn*10*d-cam insertion mutants that survived as well as wild-type bacteria in mice after oral administration. *S. typhimurium* strain 14028s was transduced with the mini-transposon Tn*10*d-cam, and a library of insertion mutants was selected on minimal media (M9) containing CM to eliminate insertion mutants of genes involved in biosynthesis of the various building blocks of the microorganism (e.g., amino acids, nucleosides, vitamins, etc). Thousands of colonies appeared on the selection plates. We picked 10 colonies for analysis of the location of the Tn*10* insertion by Southern blot. Chromosomal DNA from each of the 10 Tn*10* insertion mutants was isolated, digested with *Eco*RI and probed using a radiolabeled DNA probe specific for Tn*10*d-cam (Fig. 1A). Every hybridization band seen in Fig. 1A was of a different size, which indicated that in each mutant, the insertion site was unique, and that Tn*10* inserted randomly into the *S. typhimurium* chromosome.

The thousands of CM-resistant colonies on the selection plates were pooled and grown in M9 media containing CM (15 μg/ml) overnight. The overnight culture was diluted 40-fold and then grown for another 4 h to early stationary phase (Song *et al.*, 2004). Bacteria (input) were washed with PBS and then administered to mice (2.5×10^5 CFU) through the oral route. Three days after infection, the spleens of the mice were removed and treated with lysis buffer (0.05% Triton X-

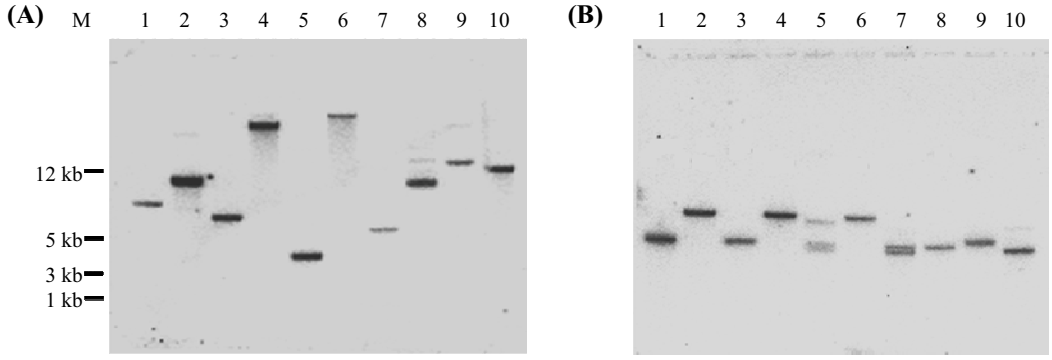

Fig. 1. Southern blot analysis of Tn*10*d-cam insertion mutants. (A) Chromosomal DNA was isolated from ten colonies from the original library of Tn*10*d-cam mutants. *Eco*RI-digested fragments were probed with radiolabeled DNA specific for *cam*. (B) Chromosomal DNA from ten CM-resistant colonies isolated from the mouse spleen after three rounds of infection was analyzed as described for (A). Numbers represent individual isolates. M indicates molecular weight size markers.

100 in PBS). The tissue lysate was plated on M9 medium containing CM (15 μg/ml). Again, several thousand colonies appeared on the selection plates. These were pooled and used for another round of infection. After three rounds of infection, we picked ten CM-resistant colonies and analyzed the transposon insertion site was analyzed by Southern blot (Fig. 1B). Unlike the pattern obtained from the input inoculum (prior to 3 rounds of infection), insertion sites in the passaged bacteria could be grouped into three types based on hybridization pattern: samples 1, 3, 7, 8, and 10 were in the first group (group 1); samples 2, 4, and 6 were in the second group (group 2); and sample 9 was in the third group (group 3). Sample 5 was eliminated from further analysis because of the presence of double bands.

These three types of Tn*10*d-cam insertion mutants were further characterized by nested PCR using the NX2 and CMR primers (Fig. 2). Bands of approximately 0.9 kb and 0.7 kb were amplified from group 1 and group 2 mutants, respectively, whereas no sequences were amplified from the group 3 mutant. These results indicated that the selected mutants arose by clonal expansion of a small number of original *Salmonella* transductants.

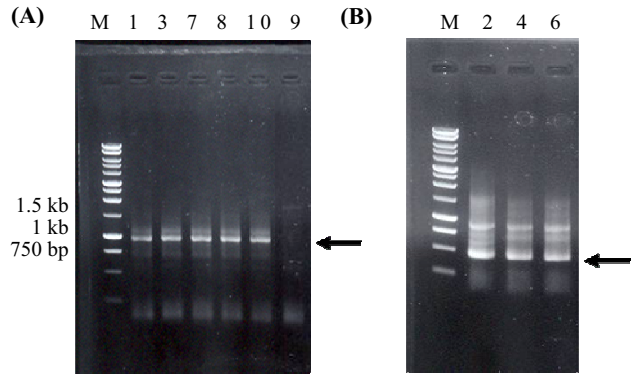

Fig. 2. Identification of Tn*10*d-cam insertion sites using nested PCR. Nine of the Tn*10*d-cam insertion mutants in Fig. 1B were characterized by nested PCR using NX2 and CMR primers. (A) Group 1 insertion mutants, showing identical 0.9 kb bands in each isolate. (B) Group 2 insertion mutants, showing identical 0.7 kb bands in each isolate. Numbers correspond to the isolates in Fig. 1B. M indicates molecular weight size markers.

The position of the Tn*10*d-cam insertion in group 1 and 2 mutants was determined by sequence analysis of the DNA fragments that were amplified by nested PCR. The Tn*10*d-cam insertion in group 1 mutants was located after nucleotide 648 in *yddG* and in group 2 mutants after nucleotide 21 in *amyA* (Fig. 3). All mutants of the same group carried a Tn*10*d-cam insertion at the identical position, which confirmed that members of each group arose from clonal expansion of a single insertion mutant. There were no distinguishing features of the DNA sequence at the positions of the insertions. *YddG* encodes an inner membrane pump and *amyA* encodes a cytoplasmic α-amylase (Raha *et al.*, 1992; Santiviago *et al.*, 2002).

Functional analysis of *yddG::Tn10* and *amyA::Tn10*

We determined the rate of host cell invasion and intracellular growth by group 1 and group 2 Tn*10* insertion mutants using RAW264.7 cells. RAW264.7 cell monolayers (5×10^5 cells) were infected with *Salmonella* (5×10^6), and then gentamicin-resistant intracellular bacteria were extracted and counted at various times after infection (Kim *et al.*, 2006) (Fig. 4). Both types of insertion mutant invaded and multiplied intracellularly as well as wild-type *Salmonella.*

The competitive index (CI) is a method in which mixed infections are used to determine the degree of virulence attenuation caused by a given mutation (Hensel *et al.*, 1997, 1998). We combined an equal number (1×10^5) of wild-type and Tn*10* insertion mutant bacteria, and administered the mixed inoculum to BALB/c mice through the oral route. *Salmonella* were recovered 3 days post-infection from the spleen and then plated on LB and LB plus CM media. The CI for *amyY::Tn10* and γ*ddG::Tn10* versus wild-type *Salmonella* was 1.876 and 0.865, respectively, which indicated that the two mutant strains did not differ significantly from wild-type *Salmonella* (Table 1). These results indicated that *yddG* and *amyA* are absolutely dispensable for *Salmonella* infection of mice.

In the current study, we used a genetic approach to identify

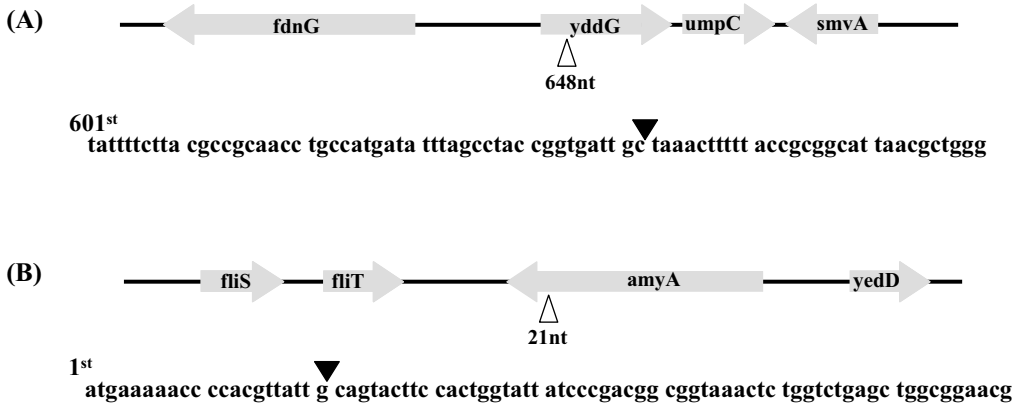

Fig. 3. Schematic of the gene targets of group 1 and 2 Tn*10*d-cam insertion mutants, sites of insertion, and flanking regions. Tn*10*d-cam insertions were located (A) after nucleotide 648 in *yddG* (group 1) and (B) after nucleotide 21 in *amyA* (group 2).

gene(s) or loci of *S. typhimurium* that are dispensable for infection of its natural host, the mouse. We identified only two genes, *yddG* and *amyA*, that do not appear to be involved in infection of the mouse by *S. typhimurium.* These findings were somewhat unexpected, given that in a previous study in which *Salmonella* mutants that failed to survive in mice were isolated by STM, most genes involved in vegetative growth of *Salmonella* in the natural environment were dispensable.

The *amyA* gene is located immediately adjacent to flagellar region IIIa, and encodes a cytoplasmic α-amylase (Raha *et al*., 1992). The deduced amino acid sequence of AmyA in *S. typhimurium* is highly similar to that of *E. coli* (87% identity). Although the physiological role of AmyA has yet to be determined, it has been shown that purified AmyA from *E. coli* digests mainly amylose, starch, amylopectin, and malto-dextrins of size G6 or larger. It is unlikely that these primary glucose reservoirs of plants are present in the internal organs of animals. Thus, AmyA likely functions only during bacterial survival outside of the animal in the utilization of carbon sources of plant origin.

In Gram-negative bacteria, a subset of inner membrane proteins in the major facilitator superfamily (MFS) act as efflux pumps to decrease the intracellular concentrations of multiple toxic substrates, thus conferring multidrug resistance (Santiviago *et al*., 2002). In *S. typhimurium*, there is a small cluster of genes consisting of *smvA*, *ompD*, and *yddG*, which encode a protein predicted to be porin, a drug/metabolite transporter, and MSF proteins that are inner membrane components of one class of multidrug resistance pump. Mutations in these genes confer increased sensitivity to methyl viologen (paraquat), and mutations in *smvA* are epistatic to mutations in *ompD* or *yddG* for this phenotype. It has been suggested that YddG and OmpD comprise an efflux pump in which the OmpD porin acts as an outer membrane channel (OMC) protein for the efflux of methyl viologen and functions independently of the SmvA pump. The open reading frames of these genes are arranged on the chromosome as follows: *smvA*, *ompD*, and *yddG*, where *smvA* is oriented toward *ompD* and *yddG*, both of which are oriented toward *smvA* (Fig. 3). It is, therefore, possible that insertional mutation of *yddG* could also prevent expression of *ompD*, if the two comprise one cistron. Nevertheless, our results indicated that the YddG-OmpD efflux pump of methyl viologen is not needed at all during *Salmonella* infection of mice. Methyl viologen is a hydrophilic, doubly-charged toxic quaternary ammonium compound that can participate in a redox cycle to generate oxygen free radicals in the bacterial cell under aerobic growth conditions. *Salmonella* during animal infection would not

Table 1. Competitive index analysis of *S. typhimurium* mutants[a]

Mixed infection	Median C.I.	No of Mice
WT versus *amyA* ::Tn*10*d	1.876	3
WT versus *yddG* ::Tn*10*d	0.875	3

[a] The competitive index (CI) was calculated as the output ratio of mutant to wild-type (WT) divided by the input ratio. CI values represent the means of three independent cycles of infection in mice. Mice were inoculated through the oral route with a mixture of two strains (1×10^5 CFU for each strain). Mouse spleens were harvested 3 days post-infection. The transposon insertion strains were differentiated on the basis of CM sensitivity. Statistical analysis was done using the Student's *t*-test.

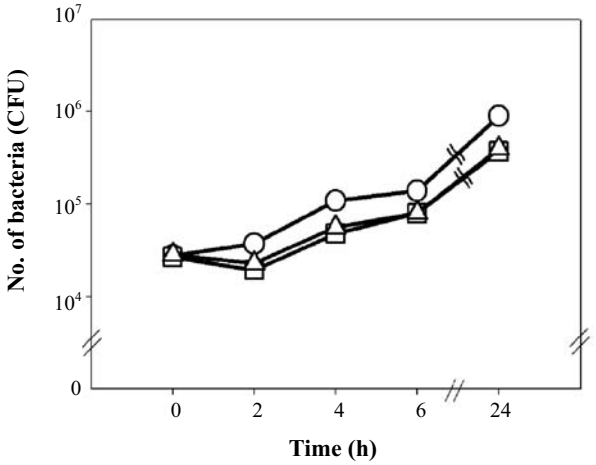

Fig. 4. Host cell (RAW264.7) invasion and replication of wild-type (14028s; ○), *amyA::*Tn*10*d (△) and *yddG::*Tn*10*d (□) strains of *Salmonella*.

likely encounter an aerobic environment with methyl viologen present. Thus, they would likely not need *yddG* for survival in the mouse. In support of this, it has been reported that there is no statistically significant difference between the LD_{50} of *S. typhimurium* wild-type and *ompD* mutant strains (Meyer *et al*., 1998).

Our results indicate that *amyA* and *yddG* are not involved in *Salmonella* infection of animals. However, it must be emphasized that in addition to virulence and virulence-associated genes, most of the genes needed for vegetative growth of *Salmonella* may be needed for optimal survival in mice. Genetic methods are likely to identify virulence genes that are absolutely required for infection and multiplication of *Salmonella* in mice (Marcus *et al*., 2000).

Virulence genes are defined as genes that encode factors or enzyme producing factors that are involved in interaction with the host and that are directly responsible for pathological damage during infection (Wassenaar and Gaastra, 2001). Virulence-associated genes encode factors or enzyme producing factors that regulate the expression of virulence genes, activate virulence factors through translational modification, processing or secretion, or are required for the activity of true virulence factors. Generally, genes involved in basic cellular metabolism ('housekeeping genes') are not regarded as virulence genes. Here we selected only two insertional mutations that were dispensable for infection of mice, which suggests that housekeeping genes also play a role, albeit perhaps not directly, in establishing pathogenesis.

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