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DNA methylation modulates *Salmonella enterica* serovar Typhimurium virulence in *Caenorhabditis elegans*

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Abstract

Salmonella enterica serovar Typhimurium was previously shown to be virulent in *Caenorhabditis elegans.* Here we demonstrate that DNA adenine methyltransferase (DAM) modulates *Salmonella* virulence in the nematode, as it does in mice. After 5 days of continual exposure to bacteria, twice as many worms died when exposed to the wild-type than the *dam*-mutant strain of *Salmonella*. Similar trends in virulence were observed when worms were exposed to *Salmonella* strains for 5 h and transferred to the avirulent *Escherichia coli* OP50. While a 10-fold attenuation was observed in the absence of DAM, the *dam*-strain was still able to infect and persist in the host worm. Our results further support the use of *C. elegans* as an accessible and readily studied animal model of bacterial pathogenesis. However, our results suggest that crucial host responses differ between the murine and nematode models. Additionally, we carried out preliminary liquid culture based experiments with the long term goal of developing high throughput animal based screens of DAM inhibitors.

Keywords: DNA methylation; Virulence; *Caenorhabditis elegans*; *Salmonella typhimurium*

1. Introduction

Studies of the free-living nematode *Caenorhabditis elegans* have provided important insights with relevance to mammals [1–3]. However, *C. elegans* has only recently been studied as a model organism in mammalian host–pathogen interactions. These studies have provided insights into the virulence mechanisms of the pathogenic organisms and have established an accessible model for studying host responses. Ausubel and coworkers [4] first used the nematode worm in solid media based assays to study host-pathogen interactions with human bacterial pathogens *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium [5,6], and with the human fungal pathogen *Cryptococcus neoformans* [7]. These studies demonstrated that mammals and *C. elegans* share similar infection-like mechanisms. In addition, liquid media studies using *Streptococcus pyogenes* [8] and *Bacillus thuringiensis* [9] showed that the release of diffusible exotoxins provides a means to express virulence.

Methylation of DNA at the N^6-adenine position, which is not found in mammals, is a primary example of an epigenetic change wherein gene expression is altered without DNA sequence modification [10,11]. Methyltransferases involve an unusual mechanism in which the target base is stabilized outside the B-form double helix during catalysis [12]. The product, N^6-methyladenosine, alters the DNA structure and affects DNA-protein interactions. Consequently, DNA

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Adenine Methyltransferases (DAMs) regulate DNA replication, methyl-directed mismatch repair, transcription, viability, and virulence in various bacteria [13]. The majority of prokaryotic DNA methyltransferases have a cognate restriction endonuclease, but a small set of MTases lack such a partner. DAM is one such enzyme, and is prevalent among enteric bacteria, including *Escherichia coli*, *Salmonella* species, *Yersinia* species, and *Vibrio cholerae*. In uropathogenic *E. coli*, DAM controls the expression of Type I pili that are required for adherence to host cells [14]. *Salmonella* DAM mutants displayed defects in protein secretion, cell invasion, and M cell cytotoxicity [15], such mutants were also unable to colonize deep tissue in a murine model and were avirulent [16]. Although DAM is not essential for viability in *E. coli* and *Salmonella*, it is required for the growth and viability of both *V. cholerae* and *Yersinia pseudotuberculosis* [17]. Since mammals exploit DNA cytosine methylation and lack any detectable DNA adenine methylation, the bacterial enzymes have become the target of antibiotic development [18].

Our objectives were to validate the use of *C. elegans* to study mammalian pathogens by demonstrating that *S. enterica* serovar Typhimurium DAM is important for pathogenesis in the nematode as demonstrated in mice, and to use this information to determine which virulence genes are regulated by DAM. Further, a major factor in drug discovery efforts is the transition from in vitro, protein and cell-based screens to those involving animal studies. Recent advances in using isogenic cell lines [19], gene array-based expression analysis [20], and various multi-mode fluorescence-based detection approaches are encouraging [21]. However, an animal model that provides an intermediate level of compound screening prior to cost- and time-intensive studies using mammals would be attractive. In particular, an animal model that provides information about lead compound efficacy and toxicity would be particularly useful. *C. elegans* provides many attractive features in this context. The molecular architecture of vertebrate and invertebrate systems, including drug metabolism and nervous systems, is mostly shared [22]. It is estimated that ~0.6% of the 16,000 known *C. elegans* genes code for cytochrome P450 enzymes, and the vast majority are closely associated to the three families of mammalian drug metabolizing enzymes [23]. While clearly not a substitute for mammalian toxicity studies, recent efforts to study both toxicity [24,25] and metabolism [22] in *C. elegans* provide support for its use as an initial, rapid and inexpensive screen.

Our experiments were designed largely to determine whether DAM modulates *Salmonella* virulence in *C. elegans*. This would further validate *C. elegans* for pathogenesis studies and provide a basis for screening DAM inhibitors. We adapted the solid media killing assay used by Ausubel [5], and a liquid assay detection procedure based on a prior method developed by Pomeroy et al. [26] (Union Biometrica).

2. Materials and methods

2.1. Strains and media

E. coli OP50, *S. enterica* serovar Typhimurium SL1344 (wild-type), and *S. enterica* serovar Typhimurium SV4392 (*dam*-) [15] were grown at 37 °C overnight in Luria-Bertani broth. Bacterial lawns used for killing experiments were prepared using modified nematode growth media (NGM) agar (0.35% peptone) seeded with 10 μl of bacteria on large 100 mm plates. Plates were incubated at 37 °C for 24 h and allowed to equilibrate to room temperature before seeding worms onto the lawns. N2 and JK509 strains of *C. elegans* were maintained in a 15 °C incubator as hermaphrodites growing on NGM while feeding on *E. coli* OP50. JK509 worms were incubated at 25 °C during experiments for sterility [27].

2.2. Plate-based worm killing assay

C. elegans N2 worms were synchronized to the L4 stage while feeding on *E. coli* OP50. NGM experimental plates (10 mm) were prepared by spreading 10 μl of bacteria into a lawn and incubating at 37 °C for 24 h. Ten worms were picked onto each plate using a metal wire loop. Each experiment was carried out in triplicate. Worms were transferred every 24 h to fresh lawns and were scored as dead when failing to respond to physical stimulus [5]. Worms that died by adhering to the Petri-plate walls were not included in the analysis. Three experiments were carried out at 25 °C, each one altering the amount of virulent bacteria being fed to the nematodes. Time required for 50% of worms to die (TD_{50}) and the associated standard error were determined by extrapolation.

2.3. Worm shift killing assay

Synchronized L4-stage N2 *C. elegans* worms (10) were fed SL1344 wild-type *Salmonella* for 5 h on a 100-mm NGM plate [5]. In the same manner, another 10 synchronized L4-stage worms were fed on a 100 mm NGM plate with SV4392 *dam*-mutant lawn for 5 h. Both experimental groups were then transferred onto *E. coli* OP50 seeded 10 mm plates and transferred every 24 h to fresh OP50 lawns. Control groups were fed on OP50 10 mm plates. Each experiment was carried out in triplicate. Worms were scored as dead when physical stimuli failed to generate any response. Worms that died by adhering to the Petri-plate walls were discounted.