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Survival of *Caenorhabditis elegans* Infected with *Escherichia coli* DFB1655 is not Affected by a Missense Mutation in *dop-1* or Treatment with Chlorpromazine Hydrochloride

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SUMMARY The O antigen producing *Escherichia coli* strain DFB1655 L9, has been shown to be pathogenic to *Caenorhabditis elegans*, with killing rates comparable to the known *Caenorhabditis elegans* pathogen, *Pseudomonas aeruginosa*. Inhibition of the dopamine signaling pathway has been found to increase survival of *Caenorhabditis elegans* infected with *Pseudomonas aeruginosa* via activation of the p38/MAPK pathway. It is not known whether loss of dopamine signaling affects *Escherichia coli* infection of *Caenorhabditis elegans*. Here, we aimed to investigate the effect of putative inhibition of dopamine signaling on the nematode survivability against *Escherichia coli* DFB1655 L9 infection. We hypothesized that successful attempts at inhibiting dopamine signaling would protect nematodes against DFB1655 infection based on previously reported results from *Pseudomonas aeruginosa* infection. Using a 10 or 12- day survival assay, we compared survival of *Caenorhabditis elegans* wild type (WT) Bristol N2 strain and a *dop-1* mutant strain of *Caenorhabditis elegans* with a missense mutation in D-1 like dopamine receptor against DFB1655 infection. Chlorpromazine hydrochloride was also used as a dopamine receptor antagonist in WT worms. Our results show that *Caenorhabditis elegans* have a significantly lower survival percentage over time when growing on DFB1655 compared to growth on the parent MG1655 strain. Moreover, *dop-1* mutant *Caenorhabditis elegans* showed no enhanced survivability compared to WT worms infected with DFB1655 L9. No increased survival over time was observed in CPZ treated WT worms compared to the water treated controls. This study establishes an *Escherichia coli* infection model in *Caenorhabditis elegans* in an undergraduate laboratory setting and confirms the previous findings that O antigen expressing *Escherichia coli* is pathogenic to *Caenorhabditis elegans.* Moreover, the data indicates that treatment of *Caenorhabditis elegans* with CPZ and a missense mutation in *dop-1* does not provide protection to DFB1655 infection.

INTRODUCTION

Caenorhabditis elegans are small, free-living nematodes that are commonly recovered from rotting fruit, temperate soils, compost heaps, and other forms of vegetation, where they directly consume bacteria for sustenance (1,2). In laboratory settings, *C. elegans* are typically grown on agar plates containing *Escherichia coli* OP50, a uracil auxotroph that facilitates controlled growth (3). The *C. elegans* life cycle consists of an embryonic egg stage, four larval stages (L1–L4), and an adult stage, collectively taking as short as 3 days to complete (1,4). Although, in times of nutrient depletion, overpopulation, or harsh temperatures, L2 larvae can enter an alternative life cycle that permits development into arrested L3 larvae known as the dauer stage (4).

Experiments that monitor *C. elegans* viability are routinely conducted on worms in the adult stage, given that they can survive for approximately 2–3 weeks before dying of senescence (1). However, ensuring that the cohort populations to be treated and tested are in

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near-identical stages is necessary. L4 larvae, which are uniquely characterized by a small, crescent-shaped patch on the worm's ventral region, can be reliably selected from a population of various stages to allow synchronized entry of worms into the adult stage. *C. elegans* also exist in two sexual forms: self-fertilizing hermaphrodites (two XX chromosomes) and males (single X chromosome) (4). Self-fertilization allows both the generation of stocks from a single hermaphroditic adult worm and the ability to uniformly maintain mutant alleles within a population (4).

The majority of bacteria that are pathogenic to *C. elegans* cause disease by infecting the intestine (1). This includes both Gram- positive (*Enterococcus faecalis, Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens*) bacteria, many of which are also pathogenic to humans under all or restricted conditions using similar virulence mechanisms (5,6,7,8,9). Therefore, *C. elegans* serve as a model for studying host–pathogen interactions (1). Previous studies have shown that *E. coli* K12 strains, which lack the O antigen (rough), are ineffective at colonizing the human intestine (10). This observation prompted Browning *et al.* to investigate whether restoration and surface expression of O antigen has an effect on the ability of *E. coli* K12 to colonize the intestine and/or mediate disease (10).

Genes in the *rfb* cluster encode the enzymes responsible for biosynthesis of the O antigen in *E. coli*. More specifically, the disruption of *wbbL* by an IS5 element, also known as the *rfb-50* mutation, renders *E. coli* K12 phenotypically rough (10). From complementation studies, it was found that *wbbL* encodes a rhamnose transferase (11). Using a suicide vector containing a functional *wbbL*, Browning *et al.* conjugated the rough *E. coli* K-12 strain MG1655 to generate *E. coli* DFB1655 L9 and *E. coli* DFB1655 L5 from two different recombination events (10). DFB1655 L9 contains an intact *rfb* cluster (10). From survival assays performed on *C. elegans*, it was demonstrated that restoration of the O antigen in DFB1655 L9 results in increased virulence compared to MG1655 and similar rates of killing as the pathogenic strain *Pseudomonas aeruginosa* PA14 (10). Browning *et al.* postulates that the O antigen protects DFB1655 L9 from maceration by the C. *elegans* pharyngeal grinder, allowing access to the intestine and subsequent rupturing from increased bacterial numbers.

In response to infection, *C. elegans* employ epithelial-based and humoral immunity, utilizing antimicrobial effectors, reactive oxygen species, and aversive learning behavior (12). The p38/MAPK signaling is a conserved innate immunity pathway involved in host responses against pathogen-mediated perturbations of core homeostatic processes in *C. elegans* (12). Recently, it has been discovered that the p38/MAPK pathway is required for the integrity of *C. elegans* intestinal barriers against toxins such as graphene oxide (13).

The dopamine signaling pathway is initiated by the interaction between dopamine and its receptors. Dopamine receptors are categorized as D1-like or D2-like, which either increase or decrease cyclic AMP respectively (14). Dopamine signaling is an important pathway influencing the development and behavior of *C. elegans*. For instance, it has been shown that dopamine interacts with D1-like receptor DOP-4 to negatively regulate worm body size and positively regulate egg-laying (15). Touch-dependent dopamine signaling, which engages mechanosensory TRP-4 channel and an additional D2-like receptor called DOP-3, has been discovered to regulate spatial pattern preferences in *C. elegans* (16). Context-dependent normal food searching behaviors in nematodes have been shown to require D1-like receptor DOP-1 signaling (17).

In addition to modulating development and behavior, the dopamine signaling pathway is involved in host responses against environmental stress and infections. In mice and human neuroepithelioma cells, microRNA-132 associated downregulation of D1-like dopamine receptor DRD-1 and 5 has been reported in response to *Toxoplasma gondii* infection (18). Dopamine signaling via DOP-1 upregulates genes involved in xenobiotic and stress responses in *C. elegans*, thereby offering protection against *P. aeruginosa* by removing infection-induced unstable proteins in epithelia (19). Recently, it was discovered that inhibiting dopamine signaling via genetic mutation or Chlorpromazine hydrochloride (CPZ)-mediated chemical repression of D1-like dopamine receptor DOP-4, results in upregulation of p38/MAPK pathway components ultimately providing protection to *C. elegans* against *P. aeruginosa* infection (20).

The objective of this study was to establish a DFB1655 L9 infection model in *C. elegans* and use this model to investigate the effects of dopamine signaling inhibition on infection. We used *dop-1(gk771250)* mutant with a missense mutation in the gene encoding D1-like dopamine receptor DOP-1. Additionally, we used CPZ to chemically inhibit dopamine signaling. We report that a missense mutation in *dop-1* and loss of dopamine signaling mediated by CPZ treatment does not confer protection against DFB1655 infection in *C. elegans*.

METHODS AND MATERIALS

Worm and bacterial strains. *C. elegans* strains were provided by Professor Don Moerman. Bristol N2 strain VC3504 was used as the WT *C. elegans* strain for this study. The *dop-1* mutant strain (F15A8.5) is a C to T missense CRISPR Cas9 knockout in the *dop-1* allele gk771250 which encodes DOP-1 isoform A (21). *E. coli* strains were acquired from Professor Ian Henderson. The *E. coli* K-12 strains MG1655 and DFB1655 L9 with *wbbL* recombination were used in this study to establish an infection model in *C. elegans*.

Preparing MG1655 and DFB1655 L9 lawns. Overnight cultures of *E. coli* MG1655 and DFB1655 L9 were grown from isolated colonies by incubating at 37°C for 16-20 hours. DFB1655 cultures were kept on 50 µg/ml of kanamycin. 50 µl of overnight cultures were spread on solid Nematode Growth Medium plates (Per 1 L dH_2O, 2.5 g peptone, 3 g NaCl, 17 g bacto-agar, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, 1 ml of 1 M KH_2PO_4, 1 ml of 5 mg/ml cholesterol solution in ethanol). Plates were incubated at 37°C for 16-20 hours to allow bacterial lawn formation. After incubation, plates were either used at 20-23°C for survival assays or were stored in 4°C fridge.

Survival assays and analysis of survival data. The method for solid NGM media survival assays was adopted as previously described (22). For each assay, 20 L4/young adult worms were placed in duplicate plates with bacterial lawns for each experimental condition. Sets of plates were incubated at 20-23°C throughout the 10 or 12 days of the assays. During days 2-10 and on a bi-daily basis, plates were taken out from the incubator and the number of live and dead worms were recorded under a dissecting microscope. The distinction between live versus dead was made based on the movement of the worms in response to physical stress delivered via gentle poking. Censorship was applied to worms that crawled up the sides of the plates and dried, worms that did not crawl off of the pick, or worms that died upon transfer. Instances of miscalculation on initial assay establishment were also considered for censorship. Live worms were transferred after each count onto new plates and all previous plates were discarded at the end of the experiment. Different trials were performed by different experimenters at different times. Variation between environmental conditions and worm handling was minimized by constantly monitoring the conditions and communicating among the experimenters. Data analysis was done by performing a Kaplan-Meier estimation and calculating mean life span using Online Application for Survival Analysis (OASIS) (23) . Significance was measured using the non-weighted log-rank test. Kaplan-Meier survival plots and bar plots were generated using R packages (24, 25, 26).

Chlorpromazine hydrochloride treatment. Chlorpromazine hydrochloride CPZ (≥98% TLC Sigma-Aldrich) was dissolved in deionized water and was applied at a 0.5 mM concentration to WT worms as previously reported (20). Sterile autoclaved dH_2O was applied to WT worms as controls. Application of CPZ and water was done by soaking the MG1655 and DFB1655 L9 lawns on NGM plates with 600 µl of 0.5 mM CPZ and dH_2O. Soaked plates were allowed to dry for 1 hour after gentle swirling to distribute the liquid on the surface of agar. After plates had dried, worms were transferred onto the plates.

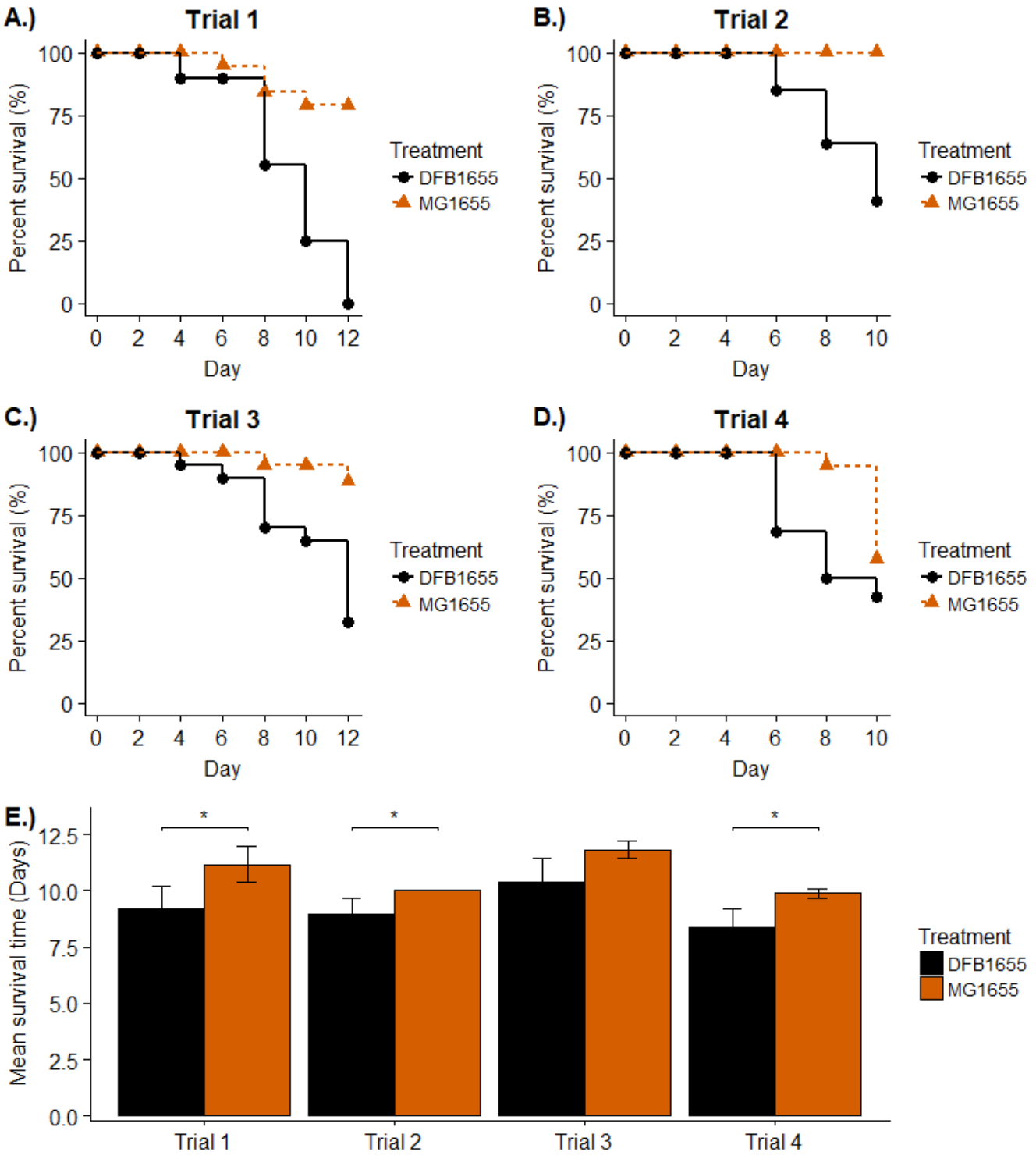

FIG. 1 DFB1655 L9 is pathogenic to *C. elegans*. (A-D) 10 and 12-day survival assays of worms growing on MG1655 and DFB1655. $p < 0.05$ for Trials 1-3. $p = 0.068$ for Trial 4. 2.5% worms were censored in Trial 1. 12.5% worms were censored in Trial 2. 22.5% worms were censored in Trial 3. 15% worms were censored in Trial 4. (E) Mean survival time for *C. elegans* growing on DFB1655 L9 and MG1655. Mean survival time calculated throughout duration of experiment where worms were followed to day 10. Value not indicative of total worm lifespan. Kaplan-Meier estimates were used to calculate mean survival times. Error bars represent 95% confidence interval. P-values for survival plots were calculated by the log-rank test. All data analysis was done by the Online Application for Survival Analysis (OASIS).

RESULTS

***E. coli* DFB1655 is pathogenic to *C. elegans*.** To test the pathogenicity of DFB1655 L9, worms were grown on control non-pathogenic MG1655 and pathogenic DFB1655 L9 for 10 and 12 days at 20^0C - 23^0C. Twenty L4-young adult worms were placed on duplicate NGM plates. The number of live and dead worms were measured periodically every two-days from day 0 until the respective end day. Worms were censored due to user error, death on the pick, or death by dehydration on the edge of plates.

Figures 1A and 1B show that *C. elegans* grown on DFB1655 L9 showed reduced survival over time compared to the WT nematodes grown on non-pathogenic MG1655 control. The same pattern of decreased survival for worms growing on DFB1655 L9 compared to worms growing on MG1655 was observed in all trials (Figures 1A-D). This pattern was statistically significant in three trials given the log-rank test (p-value < 0.05 with exception of trial 4, Figures 1A-D). The decrease in survival of worms growing on DFB1655 L9 was observed at days 4-6 of the survival assays (Figures 1A-D). There was also a statistically significantly lower mean life span for worms growing on DFB1655 L9 compared to MG1655 (p-value < 0.05 with exception of trial 3, Figure 1E). These results are consistent with the previous study showing that DFB1655 L9 is pathogenic to *C. elegans* (10).

***C. elegans* bearing a *dop-1* mutation of do not show increased survival against DFB1655 L9 infection.** To test whether the *dop-1(gk771250)* missense mutant shows increased resistance to DFB1655 infection compared to WT *C. elegans*, *dop-1* mutant and WT worms were grown on DFB1655 L9. A control group of mutant worms were grown on non-pathogenic MG1655 to determine whether or not the *dop-1* mutation affects survival of worms in absence of infection. 10-day survival assays were performed at 20°C - 23°C. Figures 2 and S2 show that *dop-1(gk771250)* mutants and WT on DFB1655 did not significantly differ in terms of percent survival through day 10 of the experiment (p > 0.05). The *dop-1(gk771250)* mutant survived on MG1655 until days 6-8, similar to a parallel experiment conducted with WT worms growing on MG1655 (compare Figure 2 with 1B-D), indicating that the *dop-1* missense mutation does not immediately affect viability of worms growing on MG1655. These results suggest that the mutation in *dop-1* does not increase survival of *C. elegans* infected with DFB1655 L9.

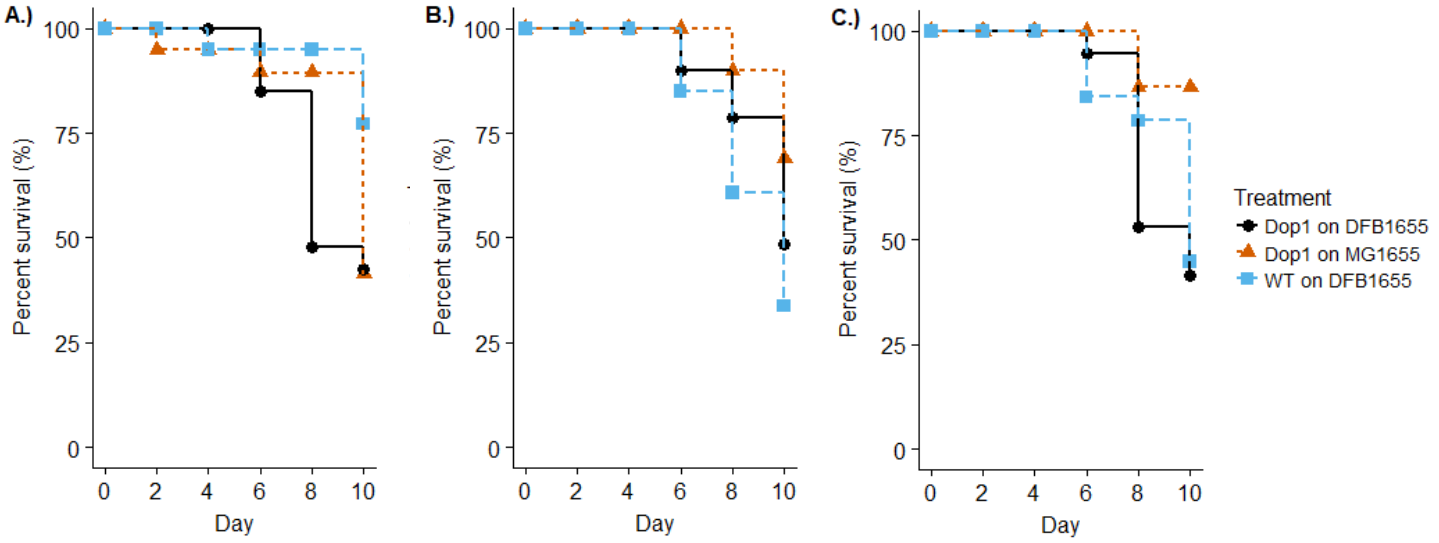

FIG. 2 Mutation of *dop-1* does not confer increased in *C. elegans* survival to DFB1655 infection. Three tirals of 10 day survival plots for *dop-1(gk771250)* mutant and WT *C. elegans* growing on DFB1655 L9 and MG1655. *dop-1(gk771250)* and WT worms grown on DFB1655 did not show a significant difference in percent survival (p > 0.05). Percent censored worms were 15%, 13.3%, and 18.3% respectively for A,B, and C. P-values were calculated by the log-rank test. All data analysis was done by the Online Application for Survival Analysis (OASIS).

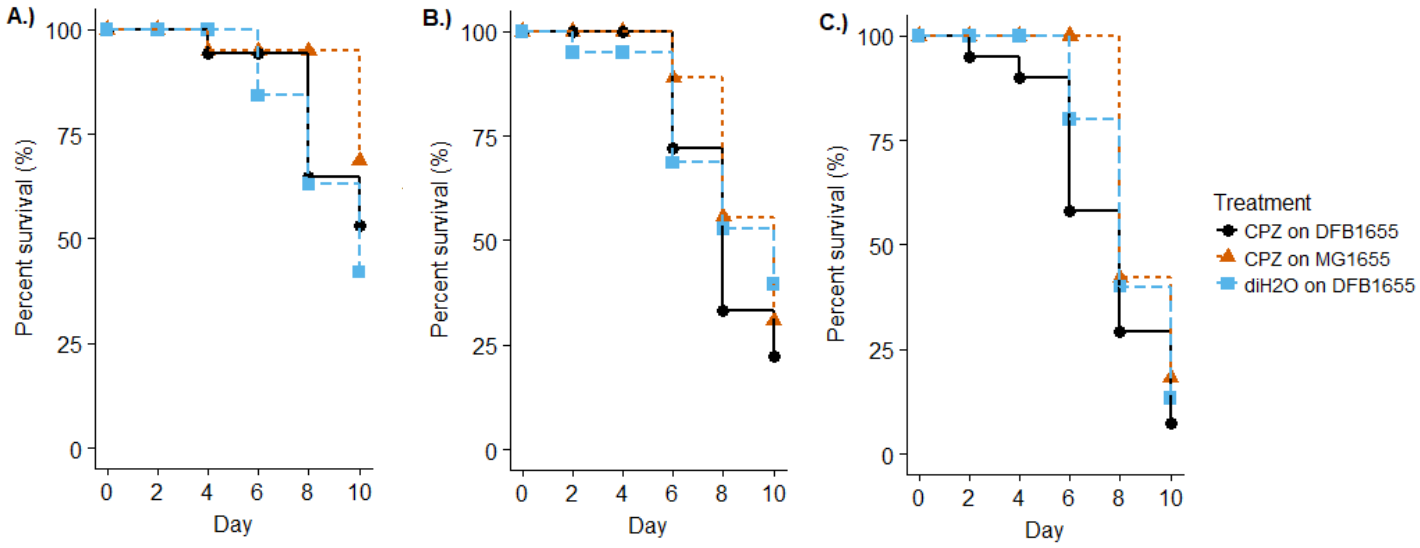

FIG. 3 Treatment with CPZ does not confer increased survival in *C. elegans* to DFB1655 infection. Representative 10 day survival plot for three trials of WT C. elegans treated with 0.5mM CPZ and dH_2O while growing on DFB1655 L9 and MG1655. CPZ and dH_2O treated WT *C. elegans* grown on DFB1655 did not show a significant difference in percent survival ($p > 0.05$). Percent censored worms were 8.3%, 15%, and 11.7% respectively for A,B, and C. P-values were calculated by the log-rank test. Error bars represent 95% confidence. All data analysis was done by the Online Application for Survival Analysis (OASIS).

CPZ treatment does *not* confer increased survival against DFB1655 L9 infection. To test whether treatment with CPZ confers increased resistance to DFB1655 infection compared to WT *C. elegans*, WT worms treated with 0.5 mM CPZ or dH_2O were grown on pathogenic DFB1655 L9. As a control, CPZ treated WT worms were grown on MG1655 to determine whether drug treatment affects survival of worms in the absence of infection. 10-day survival assays were performed at 20°C - 23°C. Figure 3 illustrates that WT worms treated with 0.5 mM CPZ had a marginally lower percent survival against DFB1655 L9 infection at all times compared to WT worms treated with dH_2O ($p > 0.05$). Figure S3 indicates that CPZ treated and dH_2O treated WT worms did not show a significant difference in percent survival ($p > 0.05$). CPZ treatment did not immediately affect the viability of worms growing on MG1655, as CPZ treated worms survived on MG1655 until days 6-8, similar to WT worms growing on MG1655 in a parallel experiment (compare Figure 3 with 1B-D). These results suggest that treating *C. elegans* with CPZ does not confer increased percent survival in nematodes against infection by DFB1655 L9.

DISCUSSION

In this study, we investigated the effect of growing *C. elegans* in the presence of the dopamine receptor antagonist Chlorpromazine hydrochloride and the effect of a missense mutation in a D-1 like dopamine receptor *dop-1(gk771250)* on survival of *C. elegans* after exposure to *E. coli* DFB1655 L9 infection. We hypothesized that treatment with CPZ and mutation in *dop-1* would protect the nematodes against infection by DFB1655 based upon previous results which elicited this effect upon infection by *Pseudomonas aeruginosa* (20). To test this hypothesis, we performed 10-day survival assays comparing *dop-1(gk771250)* versus WT worms against DFB1655 infection. We also performed another set of 10-day survival assays using CPZ treated WT worms. Even though each trial was controlled to minimize the effect of biases, this study was not double-blinded and different trials were not performed under identical conditions. As such, some variability between different trials persisted.

***dop-1(gk771250)* missense mutation does not confer protection to pathogenic DFB1655.** We did not observe a statistically significant decrease in survival in *dop-1(gk771250)* mutants versus WT *C. elegans* when grown on DFB1655 L9 ($p > 0.05$). Furthermore, no trend of increased survival for *dop-1(gk771250)* mutants compared to WT *C. elegans* was observed

among all trials (Figure 2). This suggests that the *dop-1(gk771250)* mutation does not confer increased resistance to DFB1655 L9 infection.

p38/MAPK pathway is central in the innate immune system of *C. elegans* and has been shown to be involved in the protective role of dopamine signaling inhibition against *P. aeruginosa* (12, 20). The involvement of p38/MAPK in the immune-enhancing effects of dopamine signaling inhibition was shown by upregulation of PMK-1 and *lys-2*, both of which are markers for the p38/MAPK pathway, in *dop-4(tm1392)* mutants compared to the WT (20). It is not known if markers of p38/MAPK pathway are upregulated in *dop-1* mutants. It is possible that DOP-1 signaling does not involve p38/MAPK pathway, and no immune-enhancing effect is elicited upon inhibiting DOP-1 signaling. More work is needed to discern whether DOP-1 dopamine receptor signals through the p38/MAPK pathway in the same way that DOP-4 does.

Treatment with Chlorpromazine hydrochloride does not confer protection to pathogenic DFB1655. Treatment of *C. elegans* with CPZ did not increase survival, contrary to previous findings that treatment elicited resistance to *P. aeruginosa* infection (20). The survival rates of both CPZ-treated and dH_2O-treated worms on DFB1655 were not significantly different ($p > 0.05$). No trend of increased survival for CPZ treated worms compared to dH_2O-treated worms was observed across all trials (Figure 3). These data suggest that the CPZ treatment, as previously suggested, does not confer resistance to DFB1655 L9 infection (20).

It was previously shown that dopamine signaling inhibition via CPZ treatment offers protection in *C. elegans* against *P. aeruginosa* infection (20). However, because this effect was measured in 4-day survival assays, the protection elicited by inhibition of dopamine signaling may be short-term. Since symptoms of DFB1655 L9 infection are manifested on days 4-6 of the survival assays (Figure 1A-D) and given that the CPZ treatment successfully inhibited the dopamine pathway, it is possible that the window of immune-protection was missed in our assays. More work is needed to determine the chronology of the previously characterized immune-enhancing effect of dopamine inhibition.

Decreased longevity in survival assay in *dop-1* mutant and CPZ treated nematodes. Interestingly, *dop-1(gk771250)* and CPZ-treated nematodes growing on non-pathogenic MG1655 showed decreased survival at around day 6-8 of the assay (Figures 2 & 3). This effect was observed to a lesser degree in untreated WT worms growing on MG1655 (Figures 1A-D). This observation suggests that CPZ treatment or a *dop-1* missense mutation may be involved in nematode longevity. The toxicity of CPZ to nematodes was also reported in a previous study where DMSO solutions of CPZ were found to be lethal against *C. elegans* (27). However, more work is needed to directly show the toxic effects of CPZ treatment and *dop-1* mutation, and to rule out the effect of aging by comparing the survival rates of *dop-1(gk771250)* mutants and CPZ-treated nematodes on MG1655 versus WT nematodes on MG1655.

Conclusions In conclusion, this study established *C. elegans* and DFB1655 L9 as a model for multicellular host-pathogen interactions in our laboratory and confirmed the previous findings that O antigen-expressing *E. coli* is pathogenic to *C. elegans*. We showed that WT worms growing on MG1655 exhibited enhanced survival compared to WT worms infected with DFB1655 L9. *dop-1(gk771250)* mutants showed similar rates of survival to WT *C. elegans* when infected with DFB1655 L9. Furthermore, CPZ treatment did not increase survival rates over time compared to untreated worms. Both observations are discrepant with previous studies using *P. aeruginosa*, although unexplored differences between DFB1655 L9 and *P. aeruginosa* may explain the dissimilarity. The disparity exhibited might also be due to time-dependence of the immune-enhancing effect of dopamine signaling inhibition or different signaling pathways employed by *dop-1(gk771250)* mutation.

Future Directions In our study, we were not able to show a loss of DOP-1 receptor function due to course time restraints. The discrepancy in these findings might be due to the fact that *dop-1(gk771250)* missense mutation was not confirmed to result in loss of dopamine receptor

DOP-1 function. We are not aware of any previous characterization for this mutant. More work is needed to establish *dop-1(gk771250)* missense mutant as a robust knockout model of dopamine signaling. In the future, a western blot or a functional assay can be performed to establish *dop-1(gk771250)* as a true DOP-1 knockout, and knockout mutants of the other dopamine receptors can be tested to determine the effect of dopamine receptor inhibition in protection against pathogens.

Another limitation of this study is that no pharmacokinetic data was acquired for the activity of CPZ, its optimal concentration, its half-life *in vivo* or the preferred method of drug delivery to *C. elegans*. The activity of CPZ can be tested through measuring the levels of downstream dopamine signaling effectors. Different drug concentrations or delivery methods could result in different survival patterns and challenge our findings. Pharmacokinetic data for CPZ can be collected by testing different concentrations or modes of delivery to better understand the limitations of *C. elegans* treatment with this drug. To show the decreased longevity and toxicity of dopamine signaling inhibition in worms treated with CPZ or mutated in *dop-1(gk771250)*, a survival assay can be performed with CPZ treated or *dop-1* mutated worms versus untreated WT worms growing on non-pathogenic MG1655.

To further determine whether the missense mutation in *dop-1(gk771250)* signals through the same intermediates as DOP-4 receptor, the transcriptional profile of various components of the p38/MAPK pathway can be elucidated for the *dop-1(gk771250)* mutant. PMK-1 expression can be used as a marker of p38/MAPK pathway (20). To investigate whether the immune-enhancement due to dopamine signaling inhibition is a short-term effect, the expression of different p38/MAPK components including PMK-1 can be measured during 10-day DFB1655 L9 survival assays.

Future studies may also aim to explore underlying *C. elegans* immune responses that potentially amplify the pathogenicity of DFB1655 or mediate tolerance to MG1655. Candidate immune genes could be identified using a DNA microarray to measure changes in expression levels upon treatment with DFB1655 or MG1655. Subsequent loss or gain of function experiments for candidate genes could then reveal specific host-immune mechanisms that mitigate or intensify virulence of DFB1655 and/or facilitate tolerance to MG1655.

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CONTRIBUTIONS

CJ: Figures and statistical analysis by R, Statistical analysis by OASIS, Results **OM:** Introduction, Results, Future Directions **VP:** Discussion, Results, Future Directions **AY:** Abstract, Introduction, Statistical analysis by OASIS, Materials and Methods, Results, Discussion, Future Directions

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