Investigation on various parameters influencing virulence of bacterial pathogens towards the model host *Caenorhabditis elegans*, and major mode of worm killing by different pathogens

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Abstract

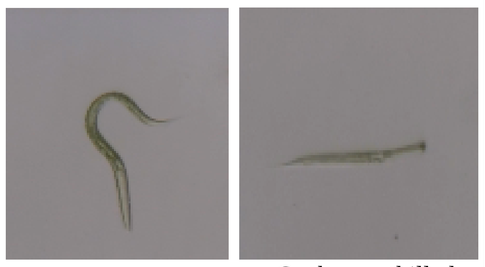
Background: *C. elegans* is widely employed as a model host for various pathogenic bacteria, while screening compound libraries for anti-pathogenic activity, as well as for studying host-pathogen interactions. However different labs may grow same pathogen in different media; and while exposing the pathogens (*in vitro*) to test compounds prior to virulence assay (*in vivo*), these compounds may be dissolved in different concentrations of Dimethyl Sulfoxide (DMSO). Hence media composition and DMSO (vehicle solvent) concentration in media becomes important factors to investigate with respect to their possible impact on bacterial virulence. **Methods**: Bacterial pathogens grown in different media (or in presence of different DMSO concentrations) were allowed subsequently (after quantifying cell density and quorum-regulated pigment) to attack *C. elegans* (in M9 buffer devoid of bacterial media or DMSO), and worm survival (live-dead count) was monitored microscopically in 24-well plates over a 5-day period. **Results:** DMSO till 3%v/v did not affect virulence of *Chromobacterium violaceum* or methicillin-resistant *Staphylococcus aureus* (MRSA), but at ≥2.5%v/v it attenuated *P. aeruginosa*'s virulence marginally. DMSO at 1-2%v/v affected growth and pigmentation, but not virulence in this pathogen. With respect to media composition, glycerol-supplementation enhanced virulence of three gram-negative bacteria namely *Vibrio cholerae*, *Serratia marcescens*, and *Escherichia coli*. Pseudomonas broth supported higher virulence in two different *P. aeruginosa* strains, while tryptone soya broth did so for two different *S. aureus* strains. Of the total 8 bacterial strains employed, 7 seemed to be dependent mainly on toxin-mediated mechanisms for killing of the worm host, while *C. violaceum* seemed to rely more on cell-mediated toxicity. **Conclusions:** While performing anti-pathogenic assays in the *C. elegans* model, sufficient attention must be paid to possible interference from various factors like, concentration of glycerol-like nutrients, or vehicle solvent like DMSO, which may influence bacterial virulence.

Objectives

1. To investigate the influence of media composition, and the common vehicle solvent DMSO on bacterial virulence, in the model host *C. elegans*.
2. To determine the major mode of worm killing by selected bacterial pathogens (cell-mediated vs. toxin-mediated killing).

Methodology

- All the reagents and media were procured from HiMedia or Merck. *C. elegans*, wild type strain (N2 Bristol) was procured from the Caenorhabditis Genetics Center, USA, and its lab food *Escherichia coli* OP50 was procured from Biovirid (The Netherlands). Worm synchronization was done as described in earlier literature (Corsi et al., 2015). Cultures of bacterial pathogens were procured from MTCC or ATCC, except *P. aeruginosa* (MDR strain) sourced from our internal collection: *Chromobacterium violaceum* (MTCC 2656), *Serratia marcescens* (MTCC 97), *Staphylococcus aureus* (MTCC 737), Methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC 43300), *Escherichia coli* (MTCC 723), *Vibrio cholerae* (MTCC 3906), and *Pseudomonas aeruginosa PAO1* (MTCC 3541). ATCC: American Type Culture Collection; MTCC: Microbial Type Culture Collection
- **Virulence assay** (Joshi et al., 2019): Bacterial pathogens were grown at 35 ºC (except 28 ºC for *S. marcescens*) for 20-24 h in different liquid growth media (with or without DMSO). Post-incubation, 100 µL of the culture broth (OD=1.50) was mixed with 900 µL of M9 buffer containing 10 worms (L3-L4 stage) in a 24-well plate (surface non-treated). This plate was incubated at 22 ºC for 5 days, and was subjected to daily live-dead counting under a microscope (4X) with halogen light source. On the last day of experiment, when plates could be opened, death was confirmed by touching the worms with a straight wire, wherein no movement was considered as confirmation of death. Standard antibiotics were used as positive controls.
- **Assay for determining mode of killing**: Overnight grown bacterial culture was centrifuged (13,500 g; 10 min). Cell pellet was resuspended in normal saline. Synchronized worms (L3-L4) in 24-well plates were challenged with 100 µL of either the bacterial cell suspension or the culture supernatant. This was followed by recording worm survival through a microscopic live-dead count over a period of five days. Standard antibiotics were employed at positive controls.
- **Statistics**: Each assay was performed as three or more independent experiments, each time in triplicate. Statistical significance of the data was evaluated by applying t-test using Microsoft Excel®. *P* values ≤ 0.05 were considered to be statistically significant.



Unchallenged *C. elegans* (live)

C. elegans, killed upon pathogen (*E. coli*) challenge

Results

Effect of DMSO on bacterial virulence

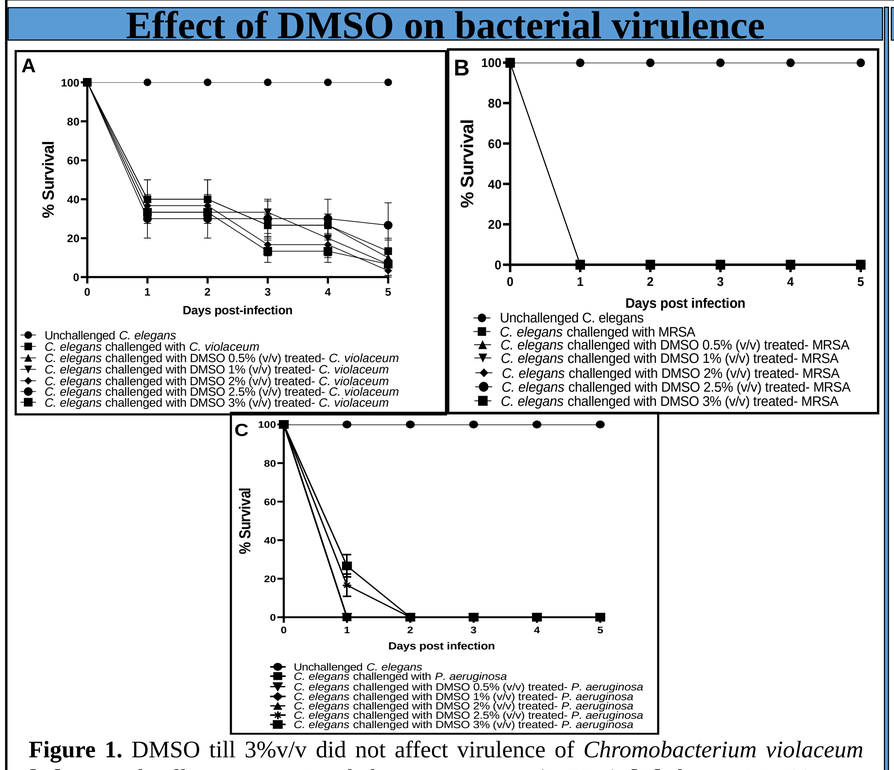


Figure 1. DMSO till 3%v/v did not affect virulence of *Chromobacterium violaceum* [A] or methicillin-resistant *Staphylococcus aureus* (MRSA) [B], but at ≥2.5%v/v it attenuated *P. aeruginosa*'s [C] virulence marginally. DMSO was tested in the range of 0.1-3%v/v, however few concentrations having no effect on virulence are not shown in these graphs to avoid overcrowding. Data pertaining to effect of DMSO on bacterial growth or quorum-regulated pigment production is not shown owing to space limitations. Wherever DMSO inhibited bacterial growth, cell densities of control and experimental bacterial cultures were equalized before the *in vivo* anti-pathogenic assay.

Effect of media composition on bacterial virulence

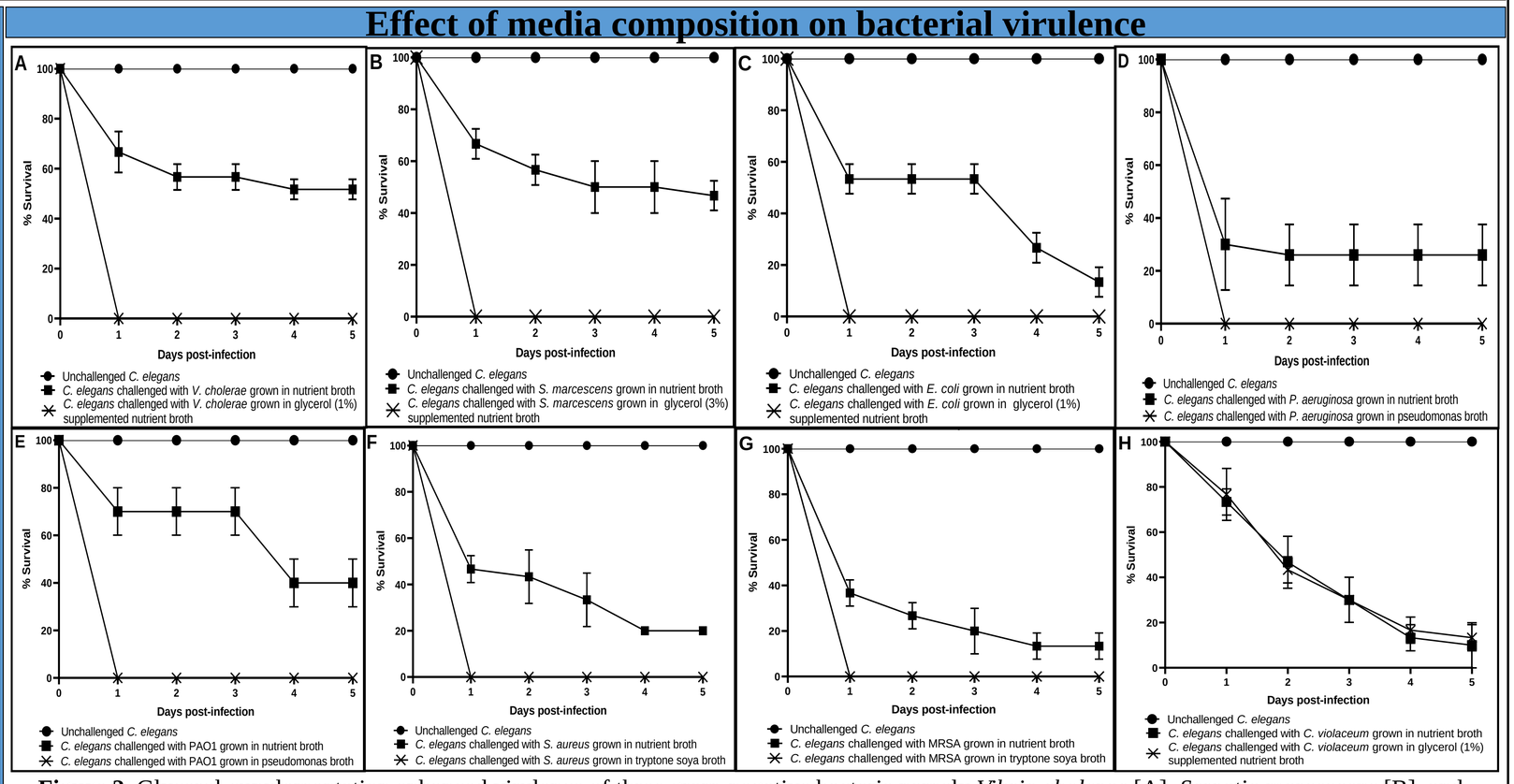


Figure 2. Glycerol-supplementation enhanced virulence of three gram-negative bacteria namely *Vibrio cholerae* [A], *Serratia marcescens* [B], and *Escherichia coli* [C]. Pseudomonas broth supported higher virulence in two different *P. aeruginosa* strains [D and E], while tryptone soya broth did so for two different *S. aureus* strains [F and G]. In all cases, where complete or near-complete killing of worm population was not achieved by bacterial culture broth, worms were able to reproduce.

Mode of killing

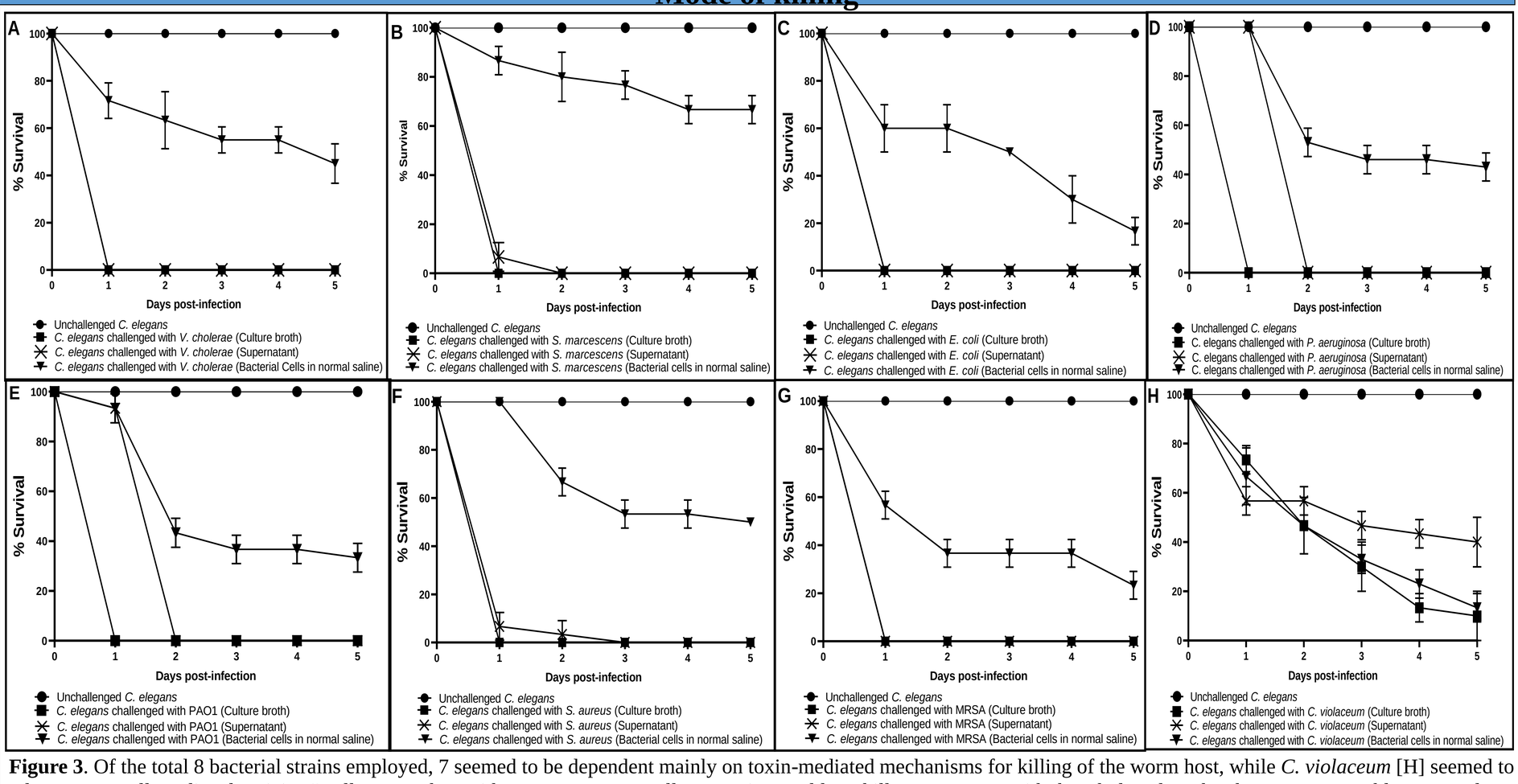


Figure 3. Of the total 8 bacterial strains employed, 7 seemed to be dependent mainly on toxin-mediated mechanisms for killing of the worm host, while *C. violaceum* [H] seemed to rely more on cell-mediated toxicity. In all cases, where either supernatant or cell suspension could not kill worms at par with the whole culture broth, worms were able to reproduce.

Conclusions

- Since DMSO is among the most widely used vehicle solvents in various biological assays, knowing its effect on bacterial virulence assumes high importance, particularly while evaluating anti-pathogenic activity of different natural products or synthetic compounds dissolved in it.
- This study has found DMSO to have marginal virulence-attenuating influence against *P. aeruginosa*. Whether it can potentiate such effect of any test compound dissolved in it, remains to be investigated.
- Media composition was also found to have significant impact on bacterial virulence. Particularly, presence of glycerol seemed to enhance the virulence of three gram-negative strains.
- Use of whole bacterial culture broth achieves faster killing without allowing progeny formation by worm population. Bacterial cells alone not only kills lesser worms than whole broth, but in their presence, worms also could reproduce, except in case of *C. violaceum*. Except the latter, all other pathogens studied displayed toxin-mediated mechanism as their major mode of host-killing.
- We conclude that use of whole bacterial culture broth is advised while performing anti-pathogenic assays employing *C. elegans* as the model host, in order to avoid interference from progenies. Further, these assays should either avoid DMSO (if test compound is water soluble), or should limit its concentration in the assay system to a level low enough to have no effect on bacterial growth, quorum sensing (Chaudhary et al., 2014) or virulence.
- Reliability of anti-pathogenic assays can be increased by selecting the media allowing optimum expression of bacterial virulence, minimizing concentration of vehicle solvents, and using whole culture as inoculum for attacking worms.

References

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