Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.7 No.2, Issue of August 15, 2004

Received November 13, 2003 / Accepted April 7, 2004
RESEARCH ARTICLE

Bacteriophage prophylaxis against *Salmonella enteritidis* and *Salmonella pullorum* using *Caenorhabditis elegans* as an assay system

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Financial support: Vice-rectoria de Investigación y Estudios Avanzados Pontificia Universidad Católica de Valparaíso. Project D.I.UCV 122.762.2001.

Keywords: animal model, bacteriophage prophylaxis, *Caenorhabditis elegans*, *Salmonella*.

The nematode *Caenorhabditis elegans* has been used as animal model system to study bacterial pathogenesis. In this study we used *C. elegans* in a bacteriophage prophylaxis assay to test phage protection against infection by *Salmonella enteritidis* and *S. pullorum*. We found that pre-treatment of nematodes with bacteriophage that lyse *S. enteritidis* and *S. pullorum* results in enhanced survival of *C. elegans*, when challenged with these bacterial pathogens.

It is useful to have cheap, manageable and reproducible animal assay systems to test aspects related to bacterial pathogenicity and its control. In this respect, the nematode Caenorhabditis *elegans* has emerged as a valid animal model. The *C. elegans* genome sequence indicates that at least 36% of 19.000 predicted proteins have a similar counterpart in humans (Sequencing Consortium, 1998). Thus, in spite of the vast evolutionary distance between nematodes and vertebrates, *C. elegans* is a valid model system to study bacterial pathogens affecting humans and other hosts. In fact, several bacterial pathogens that cause disease in humans are also able to infect and kill *C. elegans* (Couillault and Ewbank, 2002). Therefore, this nematode has been used to elucidate virulence mechanisms in *Pseudomonas aeruginosa* (Gallagher and Manoil, 2001), *Burkholderia paseudomallei* (O'Quinn et al. 2001) and *Salmonella typhimurium* (Aballay et al. 2000; Aballay and Ausubel, 2001). These last authors observed, in addition, that *S. enteritidis* is also able to infect and kill *C. elegans*.

S. enteritidis causes gastrointestinal disease in humans and is a major public health concern due to its ability to be transmitted via contaminated eggs or egg-based and poultry meat products (Dominguez et al. 2002). An approach to the control of *S. enteritidis* is the use of bacteriophage wich have proven of value in the curtailment *S. enteritidis* infection in Cheddar cheese (Modi et al. 2001) and vegetables (Leverentz et al. 2001).

In this latter context, we have recently isolated and described three dsDNA phages that lyse *S. enteritidis* in vitro; in addition, variants of these phages, that lyse *S. pullorum*, can be recovered as well (Santander and Robeson, 2002). These phage have a morphology similar to that of bacteriophage λ of *Escherichia coli.* Furthermore they form clear plaques in lawns of the aforesaid bacteria. Being phage *f*3aSE the most active we decided to test its potential use in prophylaxis, developing an assay system with *C. elegans*. *C. elegans*, as stated above, has been a valuable animal system to test bacterial pathogens with the advantages of ease of manipulation and thorough knowledge of its genetics and molecular biology. Using *C. elegans* as an assay system we found that the phage *f*3aSE protects *C. elegans* from infection and subsequent death by *S. enteritidis*. We also report that *S. pullorum* is able to infect and kill *C. elegans* and that *f*3aSP, a variant of *f*3aSE adapted to *S. pullorum*, protects *C. elegans* from *S. pullorum* killing as well.

METHODS

Bacteria and bacteriophages

Bacteria were grown in Luria Bertani medium (LB) at 37ºC. When necessary, the antibiotics (Sigma, St. Louis) rifampicin (Rif; 100 mg/ml) or streptomycin (Sm; 80 mg/ml) were added. A Rif resistant mutant of *S. enteritidis*

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ATCC (American Type Culture Collection) 13076 and a strain of *S. pullorum* (Instituto de Salud Pública-ISP-Chile) were used in infection and phage prophylaxis assays and to propagate phage. *Escherichia coli* OP50 Sm resistant (Brenner, 1974) was used to feed *C. elegans*. In addition, we used a strain of *S. enteritidis* PT4 (ISP, Chile) and *S. typhimurium* SL1344 (Aballay et al. 2000), the latter as a positive control for pathogen mediated death of *C. elegans*. The phage used were *f*3aSE, active against *S. enteritidis*, and the derivative *f*3aSP, adapted to lyse *S. pullorum* (Santander and Robeson, 2002). Liquid lysates (10^9-10^{10} pfu/ml) were prepared in LB, with aeration at 37ºC (Santander and Robeson, 2002). Phage were then washed thrice with 0.5 ml of distilled water using a 100 kD filtration unit (Eppendorf centrifugal filter tubes) and finally suspended in 0.5 ml. The suspension was titrated and kept at 4ºC.

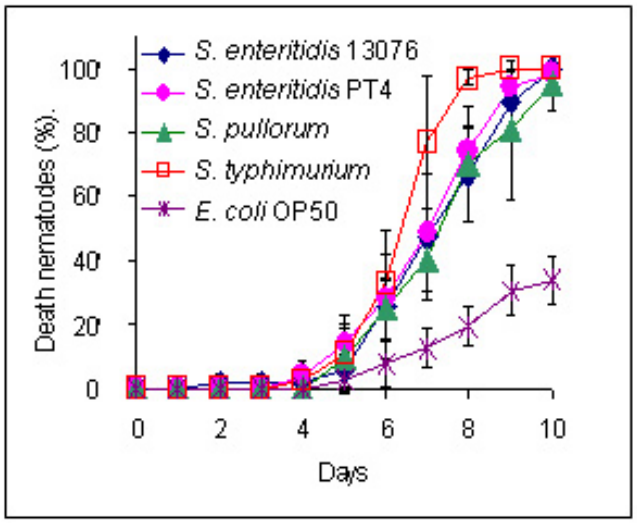

Figure 1. *C. elegans* death assay. Death of *C. elegans* caused by *Salmonella* strains. Deviations shown correspond to three independent experiments.

C. elegans maintenance

The nematode *C. elegans* Bristol N2 was maintained in modified NG agar (0.35% bacto peptone), fed with 200 ml of a fresh culture of *E. coli* OP50 per plate according to Aballay et al. 2000. For phage prophylaxis assays nematodes were fed, additionally, with 100 ml of phage suspension (10^8 -10^9 pfu/ml).

C. elegans death assay

We used the method described by Aballay et al. 2000, without transfers. Nematodes were incubated at 25ºC for 10 days. Dead nematode counts were performed every 24 hrs eliminating dead specimens from the plate. Thus, we determined the TD_{50} (Time it takes for 50% of the nematodes to die).

Presence of bacteriophage inside *C. elegans*

Nematodes fed with bacteriophage and *E. coli* OP50 were suspended in 1.5 ml of S-Basal medium (Brenner, 1974) and washed 20 times by centrifugation (10.000 r.p.m, 5 min.) in a Eppendorf 5415D centrifuge, using 1,5 ml of S-Basal per wash. The last supernatant was titrated for bacteriophage, as well as the nematode pellet after mechanical disruption with a glass pestle fitting 1.5 ml Eppendorf tubes. A control tube treated the same way, except for the mechanical disruption procedure, was used to determine total nematode counts.

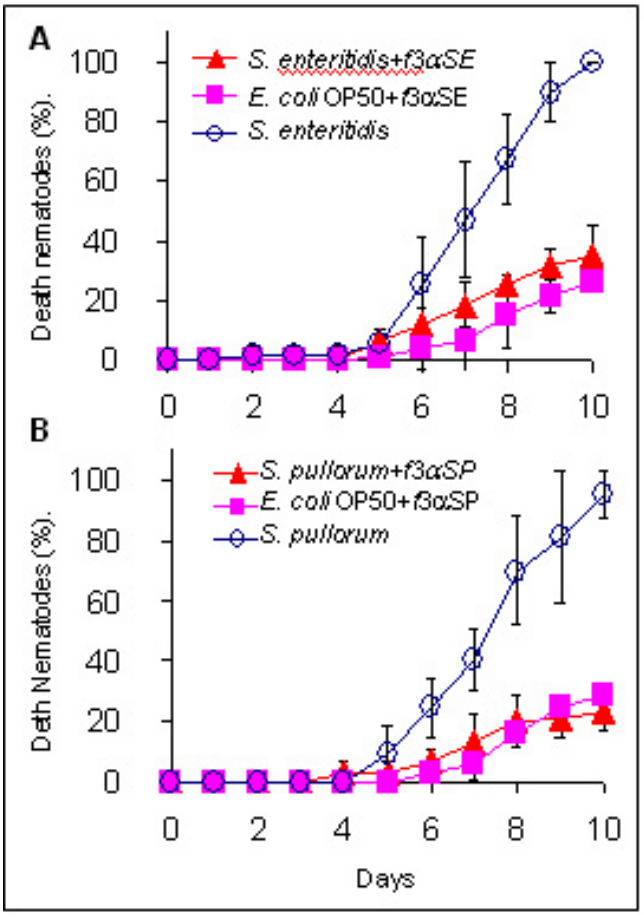

Figure 2. Assay of bacteriophage prophylaxis in *C. elegans*. A. Effect of f3aSE on *S. enteritidis* infection; **B**. Effect of f3aSP on *S. pullorum* infection. Deviations shown correspond to three independent experiments.

Bacteriophage prophylaxis assay in *C. elegans*

E. coli OP50 and bacteriophage fed nematodes kept on NG agar plates were used; 15-20 nematodes (L4 stage) were inoculated onto PGS agar plates (Aballay et al. 2000) containing pathogenic *Salmonella* bacteria. Plates were incubated at 25ºC and dead nematode counts were performed every 24 hrs.

Statistical analysis

We used one way ANOVA and Tukey`s analysis (Sokal and Rohlf, 1979) with an error factor (a) of 0.5% using a Microsoft Excel sheet. Data from the fourth to the tenth day were used (Figure 1 and Figure 2).

RESULTS AND DISCUSSION

We determined that *S. enteritidis* ATCC 13076 Rif resistant, *S. enteritidis* PT4 and *S. pullorum* kill *C. elegans* as does *S. typhimurium* SL1344 (Figure 1). The estimated TD_{50} is the same for both strains of *S. enteritidis* (*S. enteritidis* ATCC 13076, 7.02 +/- 0.72 days; *S. enteritidis* PT4 7.07 +/- 0.12 days), which were more virulent than *S. pullorum* (7.30 +/- 0.26 days). Both *S. enteritidis* and *S. pullorum* were less virulent than *S. typhimurium* SL1344 (6.40 +/- 0.25 days). These assays were performed without transfers and both eggs and larvae were eliminated, when possible, for the case of the *E. coli* OP50 grown nematodes. This was not necessary in assays involving *S. enteritidis* and *S. pullorum* since no eggs or larvae were observed. It seems that these bacteria interfere with gonad function in *C. elegans*, in a similar fashion as *S. typhimurium* does (Aballay and Ausubel, 2001).

In all *Salmonella* infection treatments, the death of *C. elegans* started at 96 hrs post-infection (Figure 1). The effects of infection were quite evident upon observation of the intestinal tract, which was dilated. This is a typical symptom shown by *C. elegans* infected by other pathogenic salmonellae, namely *S. typhimurium* (Aballay et al. 2000).

Statistical analyses showed significant differences between infection treatments and the control group of nematodes fed with *E. coli* OP50. No significant differences were observed with respect to the positive control of *C. elegans* infected with *S. typhimurium* SL1344.

In relation to the use of bacteriophage in conjunction with *C. elegans* we first determined that when the nematode was fed *E. coli* OP50 together with bacteriophage, the latter remained inside the nematode at least during 15 days as determined by their ability to form plaques (pfu) in the corresponding indicator bacteria.

For bacteriophage *f*3aSE we determined that at 15 days of incubation each nematode contains, inside, approximately 21 pfu with an initial input of 10^9 pfu per culture plate, with contains about 10^4 nematodes at the time of bacteriophage quantitation. In the case of *f*3aSP, each nematode contained, inside, about 5 pfu with initial input of 10^8 pfu/plate. It seems that the bacteriophage are innocuous for *C. elegans* since we could not observe any abnormalities in nematodes to which bacteriophage were added.

Furthermore we found that bacteriophage *f*3aSE and *f*3aSP protect *C. elegans* from killing by *S. enteritidis* ATCC 13076 and *S. pullorum*, respectively. When the nematodes containing bacteriophage are exposed to lawns of the bacterial pathogens, they showed significantly less mortality than untreated controls (Figure 2A-B). In addition, no effects were observed in the intestinal tract of bacteriophage protected nematodes.

These results suggest that phages such as *f*3aSE could be used prophylactically in chicken as an additional control measure to diminish *S. enteritidis* contamination.

In summary we have shown that *C. elegans* can be used in assays of phage prophylaxis in connection with bacterial pathogens able to kill this nematode.

ACKNOWLEDGMENTS

We thank Dr. Alejandro Aballay (Harvard Medical School) for the generous gift of *C. elegans, S. typhimurium* SL1344 and *E. coli* OP50 and Dra. Soledad Prat, ISP, Chile, for providing additional bacterial strains.

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