Experimental validation of Haldane's hypothesis on the role of infection as an evolutionary force for Metazoans

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A common drawback in evolutionary science is the fact that the evolution of organisms occurs in geological timing, completely out of the time scale of laboratory experimental work. For this reason, some relevant hypotheses on evolution of Metazoans are based on correlations more than on experimental data obtained for testing the robustness of those hypotheses. In the current work, we implement an experimental methodology to analyze the role of infections as a driving force in the evolution of Metazoans (Haldane's hypothesis). To that goal, we have used simple models of virulence with short reproduction times, large populations, and that are easily testable in the laboratory. Using the bacteriovirus nematode *Caenorhabditis elegans* as a model organism under evolution and their infection by the environmental opportunistic bacterial pathogen *Pseudomonas aeruginosa* as the selective force, we have demonstrated that bacterial infection selects an evolved nematode lineage resistant to infection, with changes in its respiration and capability of consuming novel food resources. Using an experimental approach, we show that infection is a selective force in the evolution of Metazoans as proposed earlier by Haldane.

Pseudomonas aeruginosa | *Caenorhabditis elegans* | opportunistic pathogens | experimental evolution

The role of infections in the evolution of their hosts was proposed early by Haldane. In 1932, he wrote "A study of the causes of death in man, animals and plants leaves no doubt that one of the principal characters possessing survival value is immunity to disease" (1). Later on, in 1949, the idea was stated more clearly: "the struggle against disease, and particularly infectious diseases, has been a very important evolutionary agent" (2). This hypothesis has been backed by epidemiological data showing that some human populations have a much higher than the expected percentage of some genetically inherited diseases. More recently, it has been argued that any harmful trait that is too frequent to be explained by mutation or balanced polymorphism is probably selected by infection (3). One of the first studied cases was thalassemia. It was found that there was a high prevalence of this disease in the Mediterranean region and that this phenomenon reflects heterozygote advantage against malaria (4). The demonstration of malaria resistance came on 1954, when it was described that young children with the sickle cell trait had significant fewer *Plasmodium falciparium* parasites than normal homozygotes (5). Recent works indicate that the picture is more complex than expected previously because *P. falciparium* infection might have selected changes in different human loci that contribute to resistance to the disease (6). That way, malaria could be a selective force shaping the genetic structure of human populations in the Mediterranean and other geographic regions (7).

Another relevant human disease for which an infection-driven selection has been suggested is cystic fibrosis (CF), one of the most prevalent human inheritable diseases (8). The disease is the consequence of mutations on the cystic fibrosis conductance regulator (CFTR). Because *Salmonella typhimurium* uses CFTR for entry into epithelial cells, it has been suggested that resistance to typhoid fever could be a major selective factor for the enrichment of *CFTR*-defective alleles in heterozygotes (9). More recently, the genetic basis of resistance to some infections has been established (10), suggesting a link between infection and host evolution. All of these data are in agreement with the proposed role of infection as a selective force relevant for the evolution of Metazoans. Nevertheless, in all cases, the evidence is based on the statistical analysis of already "evolved" human populations after their contact with the infective agents, and a formal, experimental evidence of Haldane's proposal is still lacking.

Although the time required for evolution is too high at the human scale for making the usual studies at the benchtop, some systems, such as bacteria and viruses, have been demonstrated to be amenable for testing evolutionary hypotheses (11) because they can change very rapidly and have extremely large populations that can be easily analyzed in the laboratory. We thus decided to test Haldane's hypothesis on the role of infections as a driving force in the evolution of Metazoans by using simple models of virulence with short reproduction times, large populations, and that are easily testable in the laboratory. One of these models is the bacteriovirous worm *Caenorhabditis elegans.* Besides being an excellent model for studies on developmental biology (12), *C. elegans* has a high mutation rate (13) and is also a very good model for studying the virulence (14–16) of several human pathogens (15). It has been stated that the effect of these pathogens on *C. elegans* is neither a "trivial consequence of the failure of the worms to ingest the microbe" nor the result of a "significant difference in 'food quality' between any of these microorganisms" (15). Different mechanisms of worm killing have been defined: infection, persistent infection, invasion, and toxin-mediated death. Those models are useful and reliable because mutations in the bacterial virulence factors required for human infection commonly challenge their pathogenesis over *C. elegans* as well (15). One of the bacteria causing death to *C. elegans* is *Pseudomonas aeruginosa* (17–19). Today this bacterial species is one of the most relevant human opportunistic pathogens (20). It is a major cause of nosocomial infections, the leading cause of infection-caused mortality in CF patients, and it has an increasingly predominant role in AIDS-associated infections. Because *C. elegans* dies upon incubation on a *P. aeruginosa* layer (17–19, 21), we have challenged *C. elegans*

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Abbreviation: CF, cystic fibrosis.

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populations with the infective clinical *P. aeruginosa* isolate PA91 with the aim of selecting a *C. elegans* mutant resistant to infection and testing whether this infection-driven selection produces relevant changes either in the physiology or in the behavior of the nematode. As the result of this approach, a mutant worm was selected by the infective pressure exerted by *P. aeruginosa*. The evolved nematode had relevant changes in its physiology and behavior, a result that validates Haldane's hypothesis by means of an experimental approach.

Results and Discussion

The wild-type *C. elegans* strain N2 was challenged with the virulent *P. aeruginosa* strain PA91 by seeding the nematode onto plates containing the bacteria under slow-killing conditions (see *Materials and Methods*). Slow killing involves an infection process and correlates with the accumulation of *P. aeruginosa* within worm intestines (19). It has been shown that many of the *P. aeruginosa* virulence factors required for killing the nematode in this assay are also required for pathogenesis in mammalian hosts. After several different challenging experiments, we detected the presence of living worms (resistant to *P. aeruginosa* infection) growing in one of the *P. aeruginosa*-containing plates. This *C. elegans* strain (AN1) was rescued, grown on *Escherichia coli* (noninfective for *C. elegans*), extracted again, and grown over a *P. aeruginosa* PA91 layer. After three passages over *E. coli*, this nematode strain was still resistant (a trait maintained at the time of writing this work, >4 years after its selection) and capable of growth with *P. aeruginosa* PA91 as its food resource. In all experiments, the susceptibility of the wild-type *C. elegans* strain N2 to *P. aeruginosa* killing was tested in parallel by using the same batch of bacterial cells. For all bacterial batches, the evolved *C. elegans* strain AN1 remained resistant and the wild-type *C. elegans* N2 susceptible to *P. aeruginosa* killing under the same experimental conditions.

The observed phenotype of resistance to *P. aeruginosa* infection is thus an inheritable trait, acquired by means of spontaneous mutation and selected because of the infection by *P. aeruginosa*. We then studied the ecological behavior (fitness) of the *C. elegans* mutant strain AN1 compared with its parental nonevolved *C. elegans* strain N2. First, both strains were grown over the noninfective food resource *E. coli* OP50 (12). As shown in Fig. 1*a*, there are no differences in productivity between both strains. Afterward, both strains were challenged with the infective bacteria *P. aeruginosa* PA91 (same batch of bacterial cells for both nematode strains). As shown in Fig. 1*b*, the nonevolved *C. elegans* strain N2 dies, whereas the *C. elegans* evolved mutant AN1 survives and is capable of successfully increasing its population size. We did not detect further changes in ecologically relevant traits such as motility in the evolved population. Together, these results indicate that the mutant *C. elegans* AN1 has a clear gain of fitness in infective conditions, and this gain does not compromise its fitness when growing over noninfective bacteria.

It has been described that clinical isolates of *P. aeruginosa* produced high levels of cyanide (22) as a secondary metabolite (23) and that cyanide can be a relevant determinant for the virulence of *P. aeruginosa* in wounds from burn patients (24) as well as in lung infections of CF patients (22). Because cyanide has also been described as a relevant element in the infection-driven killing of *C. elegans* by *P. aeruginosa* (25), we have analyzed the effect of this compound in the physiology of the wild-type and the evolved *C. elegans* strains. Three experiments on oxygen consumption, with sufficient separation in time (March 4 and April 4, 2003 and May 7, 2005), were carried out as described previously (26). At low concentrations, cyanide is an inhibitor of some oxidases of the respiratory chain, so that we measured the respiration of the nonevolved strain *C. elegans* N2 and the evolved strain *C. elegans* AN1. As shown in Table 1, the

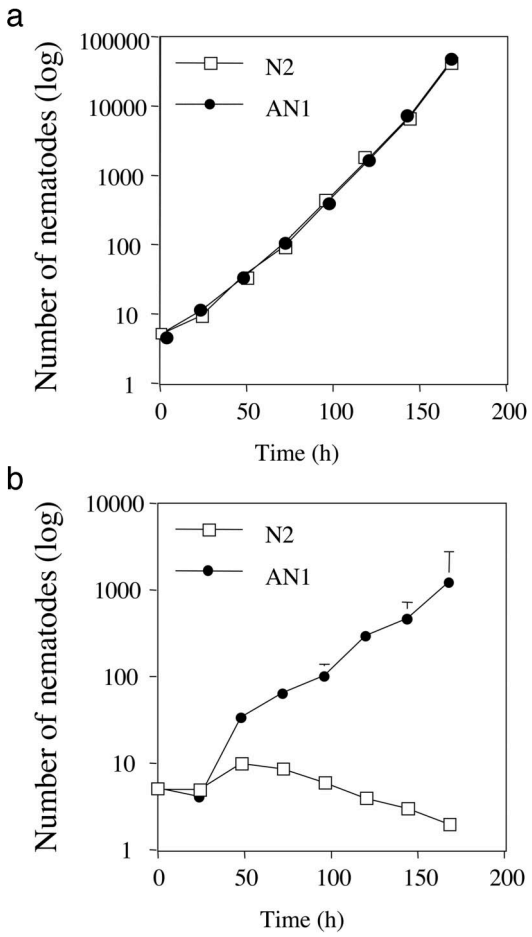

Fig. 1. Differential growth kinetics of wild-type and mutant *C. elegans* strains. Wild-type *C. elegans* N2 and its evolved derivative AN1 were grown with either *E. coli* (*a*) or *P. aeruginosa* (*b*) as its food resource. It can be seen that the mutant strain AN1 can grow (is more fit) in the presence of *P. aeruginosa* under conditions in which the wild-type line N2 is unable to multiply (*b*). Nevertheless, this gain of fitness does not compromise the fitness when both strains grew with *E. coli* as their food resource (*a*). Culture medium and general conditions were as described in the "slow-killing" assay (17, 21). Six replicates were made for each of the tested conditions, and the average values ± SD are shown. The error bars for SDs are not shown when they were smaller than the symbols used in the figure.

rate of respiration of the evolved worm *C. elegans* AN1 was lower than the rate of respiration of the nonevolved strain *C. elegans* N2, indicating a lower efficiency of the evolved strain for this specific trait. However, incubation of the nematodes with cyanide completely arrests respiration of the nonevolved nematode, whereas the evolved one keeps a proficient rate of respiration even in the presence of this poison. This finding indicates that the evolved worm can make use of either a modified or an alternative respiratory chain oxidase not inhibited by cyanide poisoning.

It is clear that infection has selected a mutant showing alterations in a highly relevant physiological trait such as respi-

Table 1. Respiration rates of *C. elegans*

Strain	Respiration per worm, pmol/h	
	−KCl	+KCN
C. elegans N2	162 ± 10	0 ± 0
C. elegans AN1	108 ± 16	29 ± 3

EVOLUTION

ration. The disequilibrium in the expected distribution of alleles of genes involved in genetically inherited diseases is the basis of Haldane's hypothesis. A trait (such as a genetically inherited disease) that reduces the fitness of the progeny can only be selected by an alternative selective force. In fact, it was suggested that the higher-than-expected frequency of these diseases in human populations is probably a side effect of the selection of lineages more resistant to infection (3). In a similar way, the selection of a *C. elegans* strain with less proficient respiration must be the consequence of an alternative selective force. In this case, the selective force is resistance to *P. aeruginosa* infection. This resistance not only allows the nematode to be more resistant to cyanide poisoning but also to gain access to a new food resource, *P. aeruginosa*. Obviously, those changes (mainly the utilization of novel food resources) may allow the evolved *C. elegans* AN1 strain to colonize novel environments previously forbidden for the wild-type strain *C. elegans* N2. At first sight, this observation might be an example of shearing evolution. The nematode will be killed as a consequence of *P. aeruginosa* infection and, besides, it will die because there is not any other food resource to feed the worm. Nevertheless, *C. elegans* can feed on nonvirulent *P. aeruginosa* cells (27), whereas mixed cultures of nontoxic *E. coli* (food resource) and *P. aeruginosa* (infective agent) kill the nematode (A.N. and J.L.M., unpublished results). This observation clearly shows that the deadly selective force for the evolution of the worm is infection, not the lack of food, and the exploitation of an alternate food resource is just an unexpected, beneficial consequence of this evolution.

Using an experimental model, we have shown here, that infection can select mutants in Metazoan organisms, with relevant changes in their physiology and ecological behavior. At the time of writing this paper, a letter strongly critical of the theory of evolution was published in a highly quoted scientific journal (28). One of the basic arguments of this letter was that the information exists in all living beings and is transferred to the offspring through "DNA can be spoiled by mutations, but never improves itself spontaneously. No positive mutations have ever been demonstrated." Here we provide a clear demonstration of this "gain-of-function mutation" in an experimental model of Metazoan evolution.

To sum up, the results shown in the present paper constitute experimental evidence of the reliability of Haldane's hypothesis on the role of infections on the evolution of Metazoans.

Materials and Methods

Bacteria and Nematode Stocks. Bacterial strains used in this work were *E. coli* OP50 (12) and the clinical *P. aeruginosa* isolate PA91. Unless otherwise specified, bacteria were grown at 37°C in brain–heart infusion (29). The *C. elegans* wild-type Bristol strain N2 used in this work was provided by the *Caenorhabditis* Genetics Center (Minneapolis, MN). The strain was maintained under standard culturing conditions at 20°C, with *E. coli* OP50 as a food source (12).

Infection-Driven Selection of an Evolved *C. elegans* Lineage. Evolution of *C. elegans* upon challenge with infective *P. aeruginosa* was performed by using the slightly modified slow-killing assay (17, 21). Briefly, a fresh culture of each bacterial strain (grown in brain–heart infusion) to be tested was layered onto a 55-mm-diameter plate containing 5 ml of potato dextrose agar (29). After spreading the bacterial culture, plates were incubated at 37°C for 24 h to form bacterial lawns. The bacterial plates were kept overnight, and worms were poured on top of these bacterial lawns. After 7 days of incubation at 24°C, the plates were scored to detect the presence of living worms. Once these evolved nematodes were selected by the selective pressure because of infection, they were incubated on nonselective medium containing *E. coli* OP50 as a food source (12). After three passages onto nonselective bacterial lawn, the evolved nematodes were challenged again with *P. aeruginosa* to ensure that the observed changes were not transient.

***C. elegans* Killing Assays.** The assays for studying bacterial killing of *C. elegans* were performed as described previously (17, 21). Bacterial lawns were grown for the slow-killing assay as described above. Nematodes were washed off the stock plates, purified by passing them through an Eppendorf microtube filled with sterile sand (12), and finally suspended in sterile distilled water. Then, each bacterial plate was seeded with five adult hermaphrodite worms. Plates were incubated at 24°C, a temperature in which worms could no longer enter into dauer lag (12). Plates were scored immediately after feeding, after 4–5 h, and each 24 h. The nematodes were examined at ×20 and ×40 magnifications. A worm was considered dead if it did not move spontaneously. In all cases, experiments were stopped after 7 days, and the nematodes were extracted from plates (when possible) by cutting the agar into small pieces. These agar pieces were soaked in water on a 90-μm sieve covered with paper tissue, and the active nematodes were recovered after 24 h (26). Each independent assay consisted of six replicates. In all cases, the *E. coli* strain OP50 was used as a control for estimating the natural death of the nematode.

Respiration of *C. elegans*. Oxygen consumption was measured mainly as described previously (26) with a Clark cell-type oxygen electrode (Rank Brothers, Ltd., Cambridge, England) with long enough separations in time (March 4 and April 4, 2003 and May 7, 2005). Experiments were conducted in a 1-ml chamber at 20°C in a physiological solution, allowing adaptation of nematodes for 1 h. When respiration became stable, 3 μl of 200 mM KCN solution was added to the chamber. Nematodes were extracted at the end of the experiment and counted under dissection microscopy.

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