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# Circadian variation in *Pseudomonas fluorescens* (CHA0)-mediated paralysis of *Caenorhabditis elegans*

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ABSTRACT

Abiotic and biotic environmental stressors play a key role in the ecophysiology of most organisms. As the presence and activity of stress-inducing agents vary along the day, organisms that are able to predict these periodic changes are better fit to survive. *Caenorhabditis elegans*, a soil-dwelling nematode, is subjected to daily changes in its natural environment, and its tolerance to osmotic and oxidative stress varies along the day. *Pseudomonas fluorescens* strain CHA0 is a soil bacterium that produces a set of secondary metabolites that antagonize phytopathogenic fungi and therefore promote healthy growth of several plant species. Here we show that strain CHA0 is able to affect *C. elegans* either under growth limiting conditions (i.e., slow-killing) or by rapid paralysis in nutrient replete conditions (fast-killing). Both types of toxicity require the post-transcriptional Gac/Rsm regulatory cascade, and the fast paralytic killing depends strongly on hydrogen cyanide production. The response observed in *C. elegans* nematodes to fast paralytic killing varies along the day and its sensitivity is higher during the night, at Zeitgeber Time (ZT) 12 (lights off). This behavior correlates well with HCN tolerance, which is higher during the day, at ZT0 (lights on). The innate immune response to *P. fluorescens* CHA0 might depend on the stress response pathway of *C. elegans*. The fact that the tolerance varies daily gives further proof of an underlying clock that governs cyclic behavior in *C. elegans*.



## 1. Introduction

Most organisms exhibit daily rhythms in their physiological variables. These rhythms are governed by a central pacemaker, composed of cellular and molecular networks, which transmit circadian time through neural/neuroendocrine signals to the whole organism. Rhythmic output is manifested through locomotor activity, the sleep/wake cycle, and various metabolic functions and physiological variables [1].

The circadian clock allows an organism to predict periodic changes in environmental conditions that might affect its viability. Therefore it represents an adaptive advantage and allows an organism to be prepared for these changes in order to survive. Several environmental cues, such as UV-light, temperature or humidity, might entrain cellular responses to environmental stressors.

*Caenorhabditis elegans* is a soil-dwelling nematode [2,3]. Previous work indicated the presence of circadian rhythms in swimming behavior [4] and response to osmotic stress [5]; moreover, this nematode exhibits some degree of homology with other clock genes well-known for other species [6]. We have recently shown a circadian rhythm of locomotor activity in this organism [7,8] and also reported that *C. elegans* exhibits daily changes in tolerance to abiotic stress [9]. Locomotor activity rhythms are entrained by the light–dark cycle and by temperature variations; moreover, they might share some common genetic pathways with other well-known molecular circadian clocks [10–12], since a *lin-42* mutant (homolog to the clock gene *period*) exhibited an abnormal circadian period. As for the response to external stimuli, it is interesting to state that in its ecological niche *C. elegans* is exposed to daily changes in both abiotic and biotic stressors. It coexists with a wide variety of microbes and feeds on them [13]; however, along their evolution, microbes have developed mechanisms to fend off nematodes. In the case of *C. elegans*, pathogenic effects can be divided into two broad categories, which are not mutually exclusive. In one, the nematode becomes infected, and presence of live microbes within the animal or attached to the cuticle is correlated with death or disease. In the other,

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secreted microbial products are responsible for symptoms [14–19].

Pseudomonads, such as *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, are ubiquitous Gram negative bacteria that, under certain circumstances, display virulence towards different organisms. Both can be found on soil samples and infection or toxins released by them might pose a biotic stress to *C. elegans*. Several studies have used *C. elegans* as a simple virulence model of *P. aeruginosa* infection [15–19]. This model presents two types of pathogenic effects on *C. elegans*. In one of them *P. aeruginosa* slowly infects the intestine of *C. elegans* and eventually kills the nematodes in a few days [18]. The other mode described involves rapid paralysis and death of *C. elegans* in a matter of hours. Depending of the culture medium used this rapid killing has been termed "Fast-killing" [15,18] or "Paralytic killing" [16]. Both types of rapid killing are mediated by toxins secreted by *P. aeruginosa* and the differences may be based on gene variations and growth conditions of distinct strains of this bacterium. The volatile hydrogen cyanide (HCN) has been identified as one of the main factors involved in the rapid killing of *C. elegans* [16].

*P. fluorescens* strain CHA0 is a soil isolate able to protect the roots of different monocotyledonous and dicotyledonous species against the deleterious effects of various pathogenic fungi, and to kill and inhibit egg hatching of the root-knot nematode *Meloidogyne incognita* [20]. These biocontrol properties depend on the production of a set of extracellular compounds including the antibiotics diacetylphloroglucinol (DAPG), pyrrolnitrin and pyoluteorin, HCN and the metalloprotease AprA [21]. Notably, the synthesis of all these products is promoted under high cellular densities by the post-transcriptional regulatory cascade Gac/Rsm that ensures coordinated metabolite secretion [22]. This cascade is mastered by the GacS/GacA two-component system that promotes transcription of three small regulatory RNAs (RsmX, RsmY and RsmZ), which bind to the translational repressor proteins RsmA and RsmE. In this way, the translational repression on mRNAs involved in extracellular secondary metabolite production is relieved in a concerted manner [22]. Thus, *gacS*, *gacA* or *rsmXYZ* mutants do not secrete antibiotics, HCN or AprA protease, and lose their biocontrol properties [23–25]. It has been recently reported that Gac/Rsm-dependent metabolite production is also important to avoid grazing by soil protists [27] and to improve CHA0 competitiveness in the rhizosphere under protist and nematode grazing pressure [28–30]. In the biocontrol isolate *Pseudomonas* strain DSS73, the Gac/Rsm system is also fundamental to escape predation by *C. elegans* nematodes [31]. Thus, biocontrol strains like CHA0 and DSS73 may represent biotic stressors to soil predators as bacterivorous protozoans and nematodes, depending on the functioning of the Gac/Rsm cascade.

In this report we have studied the effects of *P. fluorescens* strain CHA0 on *C. elegans* and showed that the rate of fast paralytic killing varies along the day. In addition we identified that Gac/Rsm-dependent HCN production is the primary factor that governs this type of nematode killing by the CHA0 strain.

## 2. Results

### 2.1. Slow-killing of C. elegans by P. fluorescens

It has been previously reported that *P. aeruginosa* exhibits two ways of killing *C. elegans* [14–19]. To determine whether *P. fluorescens* also showed those two types of killing behavior we used similar approaches to those reported. In "slow-killing" assays, we observed 50% mortality of the nematodes after 4 days of incubation for the environmental *P. aeruginosa* isolate Hex1T, after 6 days for wild-type *P. fluorescens* CHA0 and after 7 days for *P. fluorescens* CHA207 (a mutant that does not produce HCN). By contrast, the *P. fluorescens* strain CHA19 (a *gacS* mutant) did not kill the nematodes and was undistinguishable from the control plates containing the *Escherichia coli* strain OP50 used to maintain *C. elegans* populations (Fig. 1). *P. aeruginosa* strain Hex1T has been previously reported to cause slow paralytic killing of *C. elegans* [32], so it served as a control for our experiments. We found that *P. fluorescens* CHA0 is also able to kill *C. elegans* under the "slow-killing" conditions, and the *gacS* gene is required for this type of killing. The inability to produce HCN barely reduced CHA0 toxicity (Fig. 1).

To assess if bacteria were infecting the intestine of *C. elegans* during this assay we used the *P. fluorescens* strains ARQ1 and ARQ2, which are GFP-tagged derivatives of CHA0 and CHA19, respectively (Table 1). Both strains reproduced the slow-killing curves of their isogenic parental strains (data not shown). We found *P. fluorescens* ARQ1 cells, but not those of ARQ2, colonizing the intestine of the nematodes after 48 h of exposure (Supp. Fig. 1). Interestingly, intestine colonization by ARQ2 was detected much later, after 168 hs of exposure (Supp. Fig. 1D).

### 2.2. Fast paralytic killing of C. elegans by P. fluorescens

Another type of killing also exhibited by *P. aeruginosa* is known as fast paralytic killing [16]. When we assayed *P. fluorescens* for this type of behavior we found that *P. aeruginosa* Hex1T and *P. fluorescens* CHA0 both killed *C. elegans*. In this assay, the CHA0 strain resulted more aggressive than the Hex1T one (Fig. 2A). Neither *P. fluorescens* CHA19 nor *E. coli* OP50 exhibited paralytic killing of the nematodes (Fig. 2A). Again, the wild-type strain CHA0 is able to kill *C. elegans* under the "fast-killing" conditions, but the disarmed *gacS* mutant resulted innocuous for the nematodes. *P. fluorescens* strain CHA1144, a mutant that does not express the three GacS/GacA-dependent small regulatory RNAs required for activation of exoproduct synthesis, reproduced the behavior of the *gacS* mutant CHA19 (data not shown).

In colonization experiments, we found *P. fluorescens* ARQ1 cells only in the corpus of the nematode's pharynx (Fig. 3). We did not

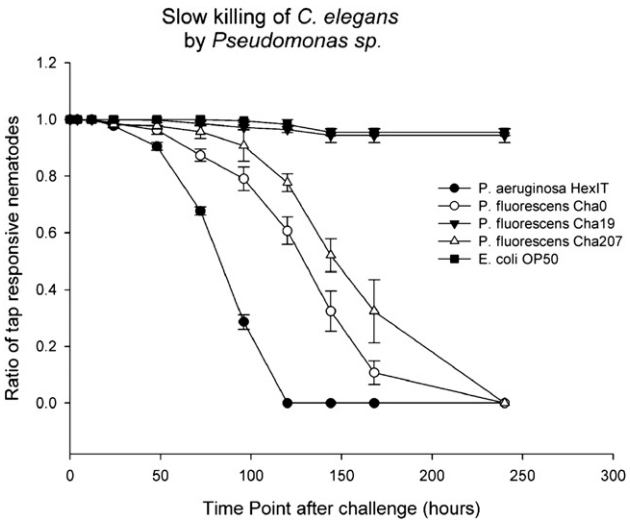


**Fig. 1.** Slow-killing of *C. elegans* by *Pseudomonas spp. C. elegans* TJ1060 day-1 adult nematodes were exposed to *P. aeruginosa* Hex1T (closed circle), *P. fluorescens* CHA0 (open circle), CHA19 (closed triangle, Δ*gacS*), CHA207 (open triangle, $HCN^-$) or *E. coli* OP50 (closed square) in slow-killing assay conditions. Worms were considered dead if they did not respond when the assay plate was tapped repeatedly against the microscope stage. Each data point represents the average level of killing based on 5 assays.