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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 [26,27] 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

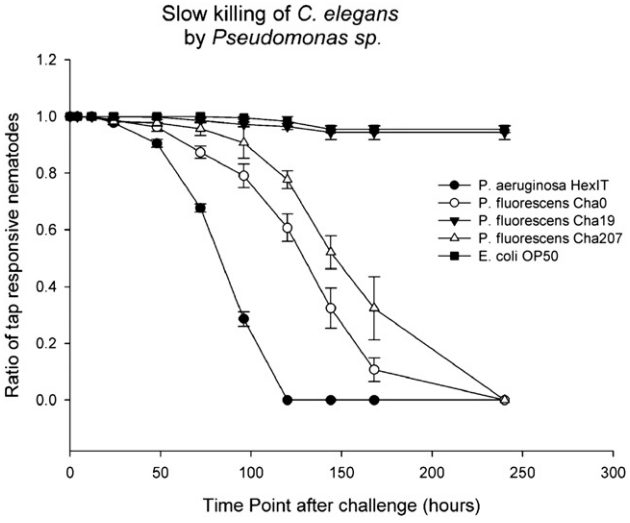

Fig. 1. Slow-killing of *C. elegans* by *Pseudomonas spp. C. elegans* TJ1060 day-1 adult nematodes were exposed to *P. aeruginosa* Hex1T (closed circle), *P. fluorescens* CHA0 (open circle), CHA19 (closed triangle, Δ*gacS*), CHA207 (open triangle, HCN^-) or *E. coli* OP50 (closed square) in slow-killing 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.

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Table 1
Strains and plasmids used in this work.

Strain		Description	Reference
C. elegans	TJ1060	(*spe-9*(*hc88*)*I*; *fer-15*(*b26*)*II*) temperature sensitive (sterile above 20 °C) mutant of *C. Elegans*	[48]
E. coli	DH5α	Laboratory strain for cloning purposes. *recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* (φ80d*lac*ZM15)	[50]
	HB101	Laboratory strain for matings. *hsdS recA proA2 leu-6 ara-14 galK2 lacY1 xyl-5 mtl-1 rpsL20 thi-1 supE44*	[50]
	OP50	Laboratory strain for maintenance of *C. elegans*. Uracil auxotroph	[51]
P. aeruginosa	Hex1T	Wild-type, isolated from hydrocarbon-contaminated soil	[52]
P. fluorescens	ARQ1	miniTn*7*–*gfp*1 at *att*Tn*7* site of CHA0; Km^R	[27]
	ARQ2	miniTn*7*–*gfp*1 at *att*Tn*7* site of CHA19; Km^R	[27]
	CHA0	Wild-type, $DAPG^+$, PLT^+, PRN^+, HCN^+, $AprA^+$	[53]
	CHA19	Δ*gacS*, $DAPG^-$, PLT^-, PRN^-, HCN^-, $AprA^-$	[25]
	CHA77	Δ*hcnABC*, $DAPG^+$, PLT^+, PRN^+, HCN^-, $AprA^+$	[24]
	CHA77+	CHA77 strain carrying the pME3013 plasmid (*hcnABC*$^+$)	this study
	CHA207	*hcnA*'–'*lacZ*, $DAPG^+$, PLT^+, PRN^+, HCN^-, $AprA^+$	[54]
	CHA207+	CHA207 strain carrying the pME3013 plasmid (*hcnABC*$^+$)	this study
	CHA631	Δ*phlA*, $DAPG^-$, PLT^{++}, PRN^+, HCN^+, $AprA^+$	[55]
	CHA1012	*pltB*::Tn*5*, Km^r, $DAPG^{++}$, PLT^-, PRN^+, HCN^+, $AprA^+$	[56]
	CHA1018	*pltB*::Tn*5*, Km^r, Δ*phlA*, $DAPG^-$, PLT^-, PRN^+, HCN^+, $AprA^+$	[56]
	CHA1144	Δ*rsmX* Δ*rsmY* Δ*rsmZ*, $DAPG^-$, PLT^-, PRN^-, HCN^-, $AprA^-$	[23]
Plasmids	pME3013	pVK100 with an 8-kb *Hin*dIII genomic fragment of *P. fluorescens* CHA0 containing the *hcnABC* genes for HCN biosynthesis; IncP replicon; RK2 Mob; Tc^R Km^R	[34]
	pME497	Mobilizing plasmid, IncP-1, Tra; $RepA^-$ (Ts); Ap^R	[47]

detect fluorescence in the intestine in fast paralytic killing assays (data not shown). Both strains, ARQ1 and ARQ2, reproduced the fast paralytic killing curves of their isogenic parental strains (data not shown).

2.3. Cyanide is one of the most important factors in *P. fluorescens* virulence towards *C. elegans*

We aimed to identify which secondary metabolite/s produced by *P. fluorescens* strain CHA0 could be responsible for the observed paralysis in *C. elegans*. We repeated the fast-killing assays using different mutants affected in exoproduct synthesis (Table 1, Fig. 2B). The mutant CHA631, unable to produce DAPG, behaved as strain CHA0, indicating the lack of DAPG toxicity in this assay. Mutants CHA1012 and CHA1018, which are deficient in pyoluteorin production, displayed a milder effect on nematodes than that of wild-type CHA0 (Fig. 2B), suggesting a partial contribution of pyoluteorin to the fast-killing phenotype. Interestingly, the HCN production deficient mutant CHA207 exhibited a markedly lower ability to paralyze *C. elegans* in the "Fast paralytic killing" assay conditions (Figs. 2B and 4). This observation was confirmed with a second HCN minus mutant (CHA77) isogenic to CHA0 (Fig. 4). We then modified the fast paralytic killing assay to test if indeed the volatile HCN was responsible for the paralysis of *C. elegans*. We did so in two ways: first, we repeated the assay but leaving the Petri dish open and found that all nematodes remained active after 4 h. Second, we modified

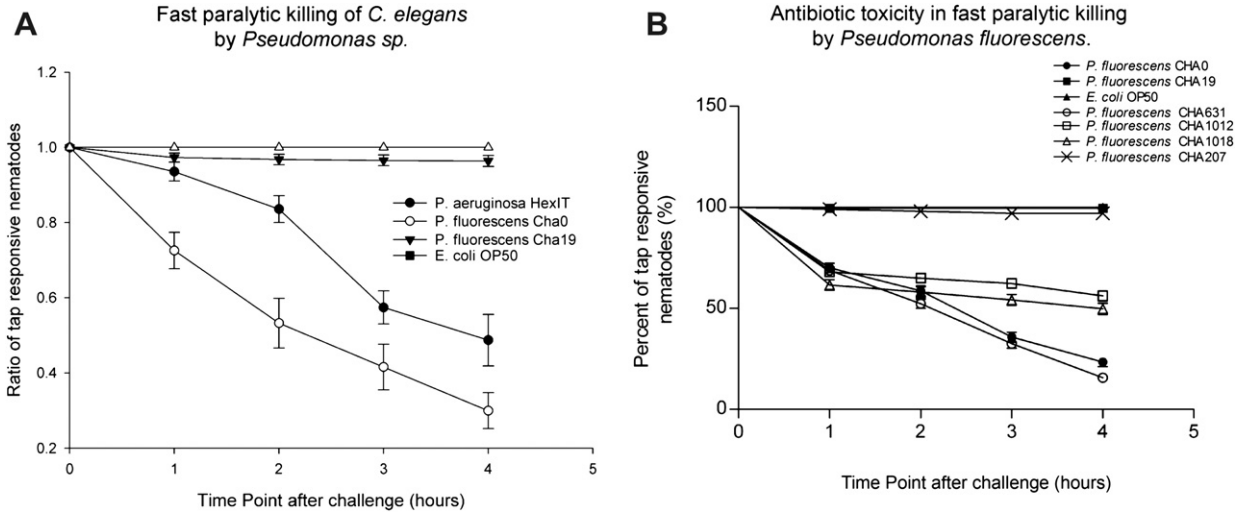

Fig. 2. A) Fast paralytic killing of *C. elegans* by Pseudomonas *spp. C. elegans* TJ1060 day-1 adult nematodes were exposed to *P.* aeruginosa Hex1T (closed circle), *P. fluorescens* CHA0 (open circle), the *gacS* mutant CHA19 (closed triangle) or *E. coli* OP50 (open triangle) in fast paralytic killing 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 at least 3 separate assays, each consisting of 3 replicates. B) Antibiotic toxicity in fast paralytic killing by *P. fluorescens*. *C. elegans* TJ1060 were exposed to *P. fluorescens* CHA0 (closed circle), the *gacS* mutant CHA19 (closed square), the HCN^- mutant CHA207 (crossed lines), the $DAPG^-$ mutant CHA631 (open circle), the PLT^- mutant CHA1012 (open square), the $DAPG^-$, PLT^- mutant CHA1018 (open triangle) or *E. coli* OP50 (closed triangle) in fast paralytic assay conditions. Each data point represents the average level of killing based on 3 separate assays.

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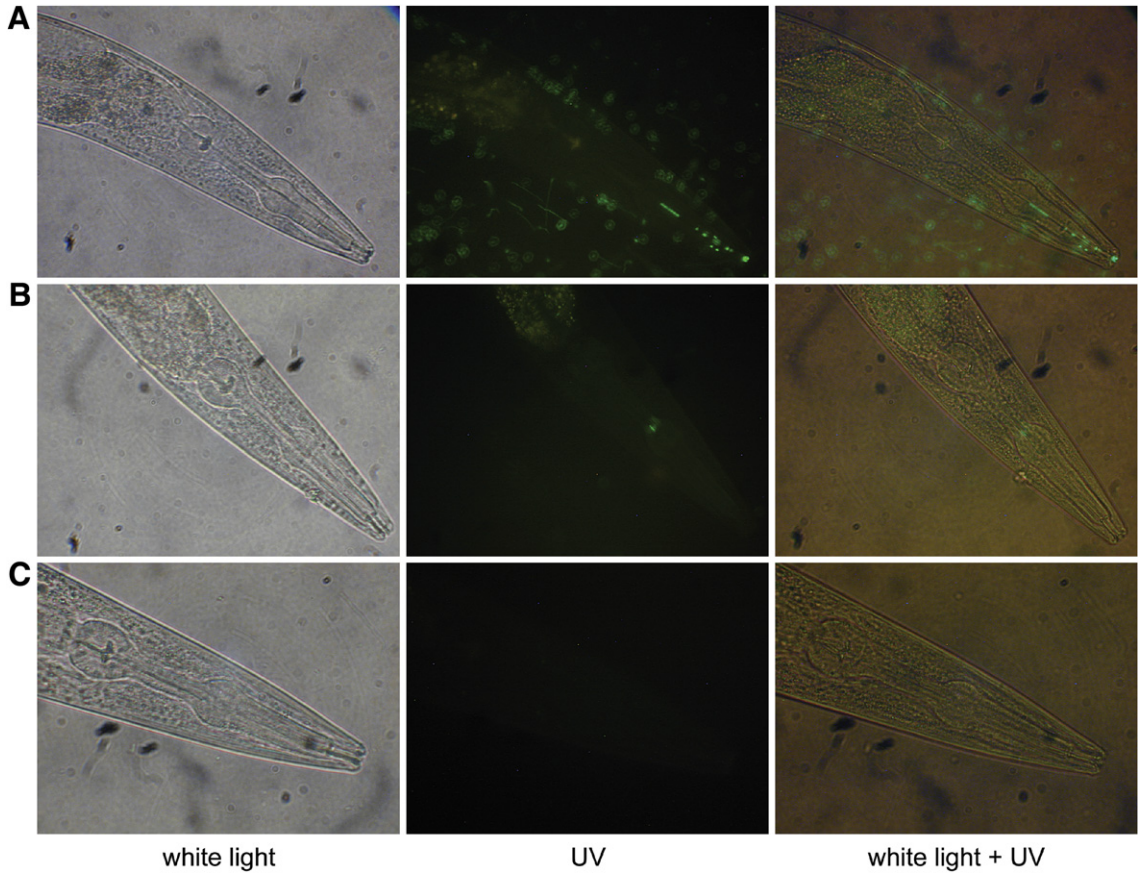

Fig. 3. *P. fluorescens* does not infect *C. elegans*' intestine under fast paralytic killing conditions. *C. elegans* TJ1060 day-1 adult nematodes were exposed to (A) *P. fluorescens* ARQ1 (GFP-tagged CHA0), (B) *P. fluorescens* ARQ2 (GFP-tagged *gacS* mutant CHA19) or (C) *E. coli* OP50, in fast paralytic assay conditions. Photographs were taken after 2 h of exposure in an optical microscope under white light illumination, with UV-light and a 490 nm filter or a combination of both light sources and a 490 nm filter. No green fluorescent emission was detected inside *C. elegans*' intestine and *P. fluorescens* cells were only detected in the nematode's corpus (pharynx) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

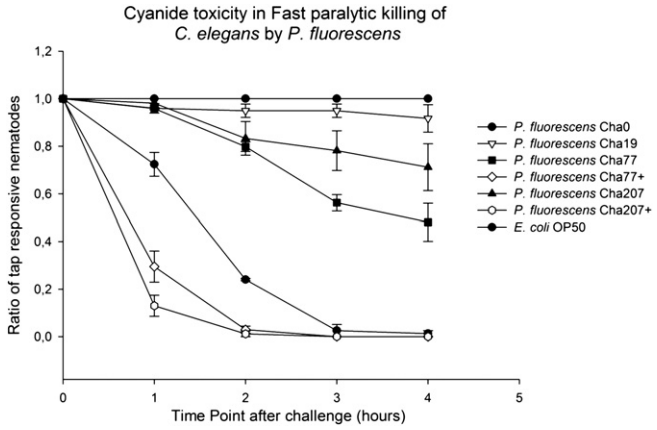

Fig. 4. Hydrogen cyanide toxicity in fast paralytic killing of *C. elegans* by *P. fluorescens*. *C. elegans* TJ1060 day-1 adult nematodes were exposed to *P. fluorescens* CHA0 (closed circle), the *gacS* mutant CHA19 (open triangle), the HCN^- mutant CHA77 (closed square), CHA77+ (open rhombus), the HCN^- mutant CHA207 (closed triangle), CHA207+ (open hexagon) or *E. coli* OP50 (closed circle) in fast paralytic assay conditions. CHA77+ and CHA207+ are chromosomal *hcn* deletion mutants complemented with a plasmid bearing CHA0 *hcn* locus. 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 at least 3 separate assays, each consisting of 3 replicates.

the killing assay by physically separating the test strain from *C. elegans* such that the contact between the test strain and the worms occurred only through the gaseous phase (see Materials and methods). We found that CHA0 was capable of paralyzing the nematodes (only 18% individuals remained active after 4 h), whereas neither CHA19, CHA207 nor OP50 induced a reduction in worm survival at the end of the experiment.

In order to further demonstrate that HCN is a major factor involved in *C. elegans* paralysis by *P. fluorescens* we complemented the two different HCN-deficient mutants, CHA77 and CHA207, with a multicopy plasmid bearing the CHA0 *hcn* operon. A colorimetric test [33] demonstrated that both complemented strains recovered HCN production (data not shown). The complemented strains, CHA77+ and CHA207+, exhibited a paralysis-inducing activity which was even higher than the one for wild-type strains (Fig. 4).

2.4. *C. elegans* tolerance to fast paralytic killing varies along the day

We have previously reported that *C. elegans* shows daily variations in stress resistance to abiotic stressors [9]. This led us to ask if *C. elegans* would also show variations in tolerance to a biotic stress such as the one posed by *P. fluorescens* CHA0 (Fig. 2). To test this, we studied "Fast paralytic killing" of *C. elegans* at two different time points: ZT0 (lights on) and ZT12 (lights off) (Fig. 5). For both *P. aeruginosa* Hex1T and *P. fluorescens* CHA0, nematodes showed a higher rate of paralytic killing during the night (Log-rank (Mantel–Cox) Test, $p < 0.0001$ and Gehan–Breslow–Wilcoxon Test,

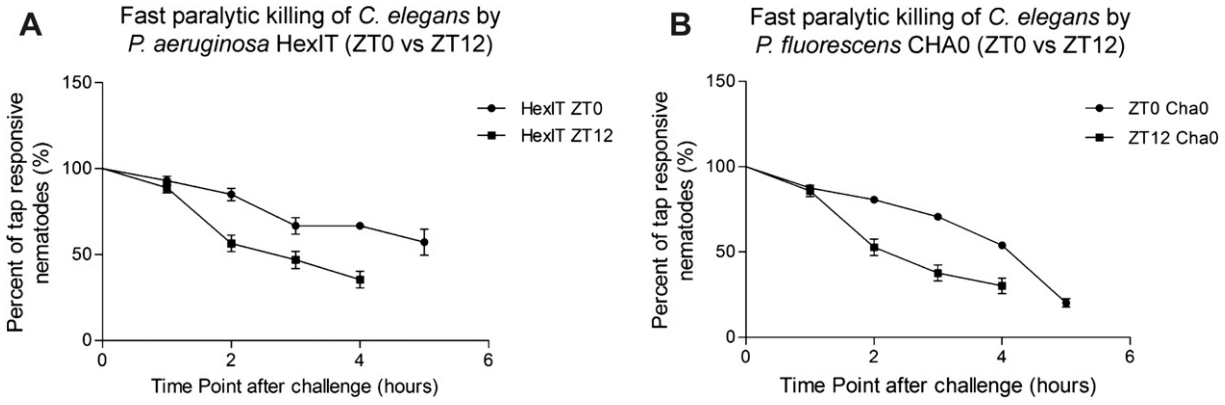

Fig. 5. Fast paralytic killing of *C. elegans* varies along the day. *C. elegans* TJ1060 maintained in LD (light:dark) 12 h:12 h conditions where subjected to a fast paralytic killing assay by *P. aeruginosa* HexIT (A) or *P. fluorescens* CHA0 (B) at ZT0 (Zeitgeber Time 0, lights on) and ZT12 (Zeitgeber Time 12, lights off). Each data point represents the average level of killing based on at least 3 separate assays, each consisting of 3 replicates. *P. fluorescens* CHA19 and *E. coli* OP50 were used as controls (data not shown).

$p < 0.0001$) (Fig. 5). This suggests a differential susceptibility to both biotic stressors along the day. *P. fluorescens* CHA19 and *E. coli* OP50 failed to affect nematode survival both at ZT0 and ZT12 (data not shown).

This diurnal change in fast paralytic killing by *P. fluorescens* was maintained under constant dark conditions (at 25.3 °C), with a peak around CT 12 (the previous time of lights off) (Log-rank (Mantel–Cox) Test, $p < 0{,}0001$), suggesting that this differential sensitivity represents a true circadian rhythm (data not shown).

2.5. *C. elegans* shows daily variations in HCN tolerance

Having found a variation in the daily tolerance of *C. elegans* to fast paralytic killing by both pseudomonads (Fig. 5) and having identified HCN as one of the major contributors to this effect (Figs. 2B and 4), we studied the tolerance of *C. elegans* to 2 μmoles of HCN at ZT0 and ZT12 and found that the nematodes could withstand exposure to HCN better during the day than during the night (Log-rank (Mantel–Cox) Test, $p < 0.0001$) (Fig. 6). Higher doses of HCN (6 or 12 μmoles) abolished the daily difference in toxicity (Supp. Fig. 2).

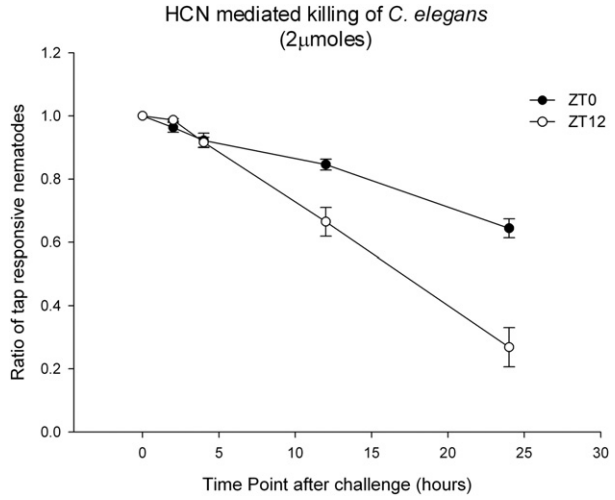

Fig. 6. HCN mediated killing of *C. elegans*. *C. elegans* TJ1060 where exposed to 2 μmoles of hydrogen cyanide gas at ZT0 (closed circle) or ZT12 (open circle). Worms were considered dead if they did not respond when the assay plate was tapped repeatedly against the microscope stage. Each data point represents the average level of killing based on 5 assays. M9 buffer was used as control (data not shown).

3. Discussion

3.1. *P. fluorescens*-mediated killing of *C. elegans*

Our results show that *P. fluorescens* strain CHA0 effectively kills *C. elegans* with a rate similar to *P. aeruginosa* Hex1T in slow-killing assay conditions (Fig. 1). GFP-tagged bacteria (*P. fluorescens* CHA0 background) were found inside the lumen of nematodes after 48 h of exposure indicating bacterial colonization. This has also been observed with *P. aeruginosa* [18] and appears to be characteristic of this type of killing. We also found that strain CHA19, which is a non-toxin producer GacS mutant, does not kill *C. elegans* (Fig. 1). This means that GacS-dependent secondary metabolites are required for slow-killing. Nevertheless, an HCN production deficient mutant, CHA207, killed *C. elegans* at almost the same rate than CHA0 (Fig. 1), suggesting that HCN is dispensable for this toxic effect of strain CHA0. As intestinal colonization was severely delayed in the *gacS* mutant *P. fluorescens* ARQ2 (GFP-tagged, CHA19 background, Supp. Fig. 1D), it appears that secondary metabolites aid in *C. elegans* gut infection.

P. fluorescens CHA0, like *P. aeruginosa* Hex1T, also shows fast paralytic killing of the nematodes (Fig. 2). It has not been yet determined what causes *P. fluorescens* to exhibit either behavior. We can only hypothesize that the medium composition appears to be a key factor to elicit one or the other type of killing. The BHI medium is a richer source of carbon and nitrogen than the NG medium, hence the bacterial density achieved in the BHI medium is higher. This, in turn, is critical for the production of secondary metabolites in *P. fluorescens* CHA0, such as extracellular enzymes, antibiotics and HCN, as its production is upregulated by a quorum sensing-like regulatory cascade mastered by the GacS/GacA two-component system [22]. In fact, the fast paralytic killing of nematodes displayed by CHA0 was abolished in a *gacS* mutant (Fig. 2).

From our data, it seems that HCN is the main or one of the most important factors involved in fast paralytic killing (Figs. 2 and 4). HCN production deficient mutants fail to kill nematodes as effectively as the wild-type strain, and when complemented with pME3013 (a multicopy plasmid containing the *hcn* operon), these mutant strains regain a higher than wild-type lethality. This can be explained by the fact that the plasmid we used to complement the strains has been previously shown to increase HCN production 5-fold relative to wild-type [34]. Our results are in agreement with a previous report that indicates that HCN is the main responsible factor in fast paralytic killing by *P. aeruginosa* [16]. There is, however, a different requirement of the GacS/GacA regulatory system for HCN mediated paralysis between *P. aeruginosa* and

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P. fluorescens. In *P. aeruginosa* fast-killing is not affected by *gac* mutations [18]; in contrast, we observed a strict requirement of the Gac/Rsm cascade for fast-killing in *P. fluorescens* strain CHA0 (Fig. 2). We think that this is due to the differential regulatory control that the GacS/A system exerts on *hcn* operon expression. Whereas a *P. aeruginosa gacA* mutation causes a 3-fold reduction in HCN production [35], *P. fluorescens* CHA0 *gac* mutants produce 25-fold less HCN than the wild-type [36].

Interestingly, a recent study shows that *P. fluorescens* CHA0 kills subterranean termites, *Odontotermes obesus*, by inhibiting the cytochrome C oxidase (CCO) of the respiratory chain. The inhibition of the CCO was dependent on the amounts of HCN produced. In this report, it was also shown that the deletion of the *hcn* operon in strain CHA77 strongly reduced termite lethality [37]. However, we cannot rule out the fact that HCN might not be the sole factor responsible for *C. elegans* fast paralysis and that some synergy might occur with some of the other secondary metabolites produced by *P. fluorescens*. Pyoluteorin also seems to be involved in the process since two pyoluteorin minus mutants, CHA1012 and CHA1018, showed a slightly lower ability to kill *C. elegans* as compared with CHA0 (Fig. 2B). Our experiments support the idea that *P. fluorescens* effectively kills *C. elegans* in a similar manner to that previously reported for, *P. aeruginosa*. However this should not be taken as a general rule for pseudomonads. *Pseudomonas* spp. strain *DSS73* does not kill *C. elegans* and it also helps protect it from killing by the flagellate *Cercomonas* in microcosm experiments [31].

The toxins released by secondary metabolism of *P. fluorescens* CHA0 effectively fend off nematode predation. This is in accordance with microcosm experiments [30] in which nematodes where placed with mixed populations of *P. fluorescens* CHA0 and the non-toxic *gacS* mutant CHA19. *C. elegans* showed preference towards preying the mutant strain. However, in this scenario CHA19 survives by improving their fitness by profiting from the exoproducts of coexisting wild-type bacteria. Feeding from the non-toxic bacteria reinforces toxin production by increasing the population frequency of CHA0. This might explain why bacteria with mutated *gacS*/*gacA* genes are frequently found in nature [38,39]. Toxin production is an efficient strategy not only to avoid predation but to increase predation on other rhizobacteria that might appear more palatable, therefore increasing competitiveness [29].

3.2. Daily variations in fast paralytic killing by P. fluorescens

It has been established that *C. elegans* responds to infections through different signaling pathways: the DBL-1 pathway, the DAF-2/DAF-16 pathway, the MAP kinase pathway and the ERK pathway, as well as through recognition of infection by the stress response [40]. In this work we showed that HCN is the major factor involved in the paralytic killing of *C. elegans*. As we have previously demonstrated that *C. elegans* shows daily variations in tolerance to abiotic stressors [9], we hypothesized that there would also be daily variations in tolerance to the biotic stress exerted by *P. fluorescens* and *P. aeruginosa*. Indeed, this was the case: we found that the rate of paralysis of the nematode population was higher during the night, at ZT12 (lights off). The ecological significance of this diurnal variation in stress resistance remains to be established. A recent report states that differences in the expression of metabolism and defense functions may in part drive nematode community dynamics through interactions with their bacterial environment [41]. The authors found that exposure to different soil bacterial food sources induces differential gene expression in *C. elegans*. Interestingly, the exposure to *Pseudomonas* spp. alters the expression of innate immunity, defense and metabolism-related genes in *C. elegans* [41]. Daily changes in gene expression could then represent an adaptation to daily changes in bacterial abundance or activity and might also contribute to soil community dynamics.

3.3. Hydrogen cyanide toxicity varies between day and night

To further understand if the daily variations observed in fast paralytic killing were due to an underlying differential stress response to HCN we assayed *C. elegans*' tolerance to different concentrations of HCN at two time points, ZT0 (lights on) and ZT12 (lights off). We found that the nematodes showed a higher sensitivity to HCN at dusk (ZT12), as was the case in the fast paralytic killing assay. However, our data also shows that at higher concentrations of hydrogen cyanide this daily variations are abolished (Fig. 3) indicating that the response varies along the day only below a certain threshold, above which the differential sensitivity to HCN disappears. Indeed, circadian rhythms in response to a toxic compound are usually evident within a range of doses. If the dose exceeds a certain limit then assays performed during the day or night show a similar survival curve. This is because the dosage used far exceeds the capability of the circadian system of the organism.

3.4. Concluding remarks

This work shows that *P. fluorescens* CHA0 presents at least two modes of killing *C. elegans*. One of them, slow-killing, requires the GacS pathway to be active but does not depend on HCN production. The other one, that resembles the fast paralytic killing mediated by *P. aeruginosa*, also requires the GacS pathway and is strongly dependent on HCN production, although other factors might also contribute to the observed pathogenicity.

Circadian rhythms in the innate immune response have been previously described in different species. One recent report shows that when the fruit fly, *Drosophila melanogaster*, is infected by *P. aeruginosa*, the survival rates of wild-type flies vary as a function of when they are infected, peaking in the middle of the night [42]. The innate immunity response observed in *C. elegans* might involve the stress response pathway [40]. It is known that the response to HCN involves the EGL-9 protein that in turn regulates the hypoxia induced factor HIF-1 [43]. It was previously reported that *egl-9* mutants are resistant to *P. aeruginosa* [15] and this could be because these mutants present a higher resistance to hypoxia. The daily variations observed in the rate of paralytic killing might represent rhythmic variations in the stress response pathway. We have previously demonstrated the existence of daily variations of tolerance to osmotic and oxidative stress. Tolerance to osmotic stress showed a maximum at ZT12 (lights off). In the field, as temperature rises, desiccation increases and a higher tolerance to osmotic stress would be expected during the day. Oxidative stress tolerance peaks at ZT0 (lights on). Reactive oxidative species (ROS) could be generated by exposure to UV-light and by the mitochondria. In *C. elegans* ROS should be increased when the nematodes exhibit higher rates of activity. We and others have shown that *C. elegans* are more active during the late night/dawn hours [4,7,8] under LD conditions, which correlates well with our results. Changes in the tolerance to stressors are orchestrated in other organisms by a central pacemaker. This might also be the case in *C. elegans*. The circadian behaviors we observed are not what is known as "masking" (an effect due to an environmental cue but not maintained by the endogenous clock) since we found that the differences are maintained in constant conditions. In LD conditions, light entrains the physiological response of the nematodes but how this happens remains unknown. Recently, it has been reported that *C. elegans* has photoreceptors [44–46] but the link between them and the circadian system remains elusive. How *C. elegans*' clock

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works and which are the mechanisms that drive it are questions that still remain to be answered.

4. Materials and methods

4.1. Bacterial strains and culture conditions

The *E. coli* and *Pseudomonas* strains used in this work are listed in Table 1. The growth media used were brain heart infusion (BHI) agar, NG agar, nutrient agar, LB broth and BHI broth. When required for plasmid maintenance, media were supplemented with 125 μg of tetracycline per ml (for *P. fluorescens*) or 40 μg of tetracycline per ml (for *E. coli*). Routine incubations temperatures were 28 °C for *P. fluorescens* and 37 °C for *E. coli* and *P. aeruginosa*. *P. fluorescens* was grown at 35 °C to improve its capacity to accept heterologous DNA in triparental matings. For complementation of the *hcn* mutants, plasmid pME3013 from *E. coli* DH5α was mobilized into strains CHA77 and CHA207 in triparental matings using *E. coli* HB101/pME497 as the mobilizing strain [47]. Chloramphenicol was used at a concentration of 20 μg per ml to counterselect *E. coli* in mating experiments. *P. fluorescens* conjugants appeared after 48 h of incubation and were checked for purity and recovery of the HCN production phenotype.

4.2. Nematode strain and culture conditions

The C. elegans strain used in this work was TJ1060, a spe-9 (hc88)I; fer-15(b26)II mutant derivative of N2 [48] (Caenorhabditis Genetics Center) (in order to maintain a homogenous adult population to perform the assays, we used this strain which is a temperature sensitive strain, sterile above 20 C). Nematode stocks were maintained in 10-cm-diameter NGM agar plates, containing an overnight lawn of *E. coli* OP50, at 16 °C in 12 h:12 h light:dark [43] photoperiodic cycles. In order to obtain synchronous day-1 adult nematodes for each assay, nematodes were transferred to 4 NGM agar plates and grown for 4 days at 18.5 °C in 12 h: 12 h LD and then synchronized by the chlorine method [49] on the 5th morning. Eggs were resuspended in 2 ml of M9 buffer + 20 μl GIBCO antibiotic antimycotic into a 50 ml erlenmeyer flask and incubated overnight at 18.5 °C, 105 rpm in 12 h:12 h LD conditions. On the 6th morning L1 stage nematodes were counted and approximately 3000 nematodes were transferred to individual 10-cm-diameter NGM plates coated with *E. coli* OP50. These plates were cultured at 25.3 °C in 12 h:12 h LD conditions until nematodes reached the day-1 adult stage.

4.3. Nematode "fast" paralytic killing assay

Paralytic killing assays were carried out in 6-cm-diameter BHI agar plates containing 10 ml of BHI agar. Overnight *P. fluorescens* BHI cultures were diluted 1/50 in BHI and used to spread 50 μl onto each plate. After incubation at 28 °C for 24 h, day-1 adult nematodes from NGM plates were collected in M9 buffer, and a 50-μl aliquot (containing 20 to 100 adult animals) was spotted onto the *P. fluorescens* lawn. The plate was then incubated at 25.3 °C with the lid sealed with Parafilm ("sealed"), or without the lid ("open"). Paralytic nematode killing was scored under a dissecting microscope every hour for 4 h. Worms were considered dead if they did not respond detectably to tapping of the assay plate against the microscope stage [15]. Fully paralyzed animals fail to respond to mechanical stimuli in randomly chosen nematodes after the assay.

4.4. Nematode "slow" killing assay

Slow-killing assays were carried out by spreading 150 μl of a 1/50 dilution in BHI broth of an overnight culture of a *P. fluorescens* colony suspended in BHI broth onto a 10-cm-diameter NG agar plate containing 15 ml of NG agar. After the plate was incubated for 24 h at 28 °C, day-1 adult nematodes from plates were collected in M9 buffer, and a 50-μl aliquot (containing 40 to 100 adult animals) was spotted onto the *P. fluorescens* lawn. The plate was then incubated at 25.3 °C with the lid on and left unsealed, and nematode killing was scored with a dissecting microscope. Worms were considered dead if they did not respond detectably to tapping of the assay plate against the microscope stage. Fully paralyzed animals fail to respond to mechanical stimuli in randomly chosen nematodes after the assay.

4.5. Volatile exoproducts killing assay

Nematodes were placed on a 3.5-cm-diameter BHI agar plate containing an overnight *E. coli* OP50 lawn. The plate with the nematodes was left uncovered and placed into a 10-cm-diameter BHI agar plate containing an overnight lawn of a specific *P. fluorescens* strain. Only volatile compounds produced by *P. fluorescens* grown around the nematode plate would hence be capable of reaching the nematodes inside the 3.5-cm-diameter plate. Once the nematodes were placed onto the *E. coli* OP50 lawn, the 10 cm-diameter plate was sealed with Parafilm and nematode paralytic killing was assessed as described above.

4.6. HCN killing assay

Nematodes were placed on a 3.5-cm-diameter BHI agar plate without its lid, and placed into a 10-cm-diameter empty plate. An inverted 3.5-cm-diameter lid containing separated aliquots of 0.18 M HCl and a defined amount of KCN dissolved in 0.09 M NaOH, was also placed within the 10 cm-diameter plate. After sealing the 10-cm-diameter plate with Parafilm, the HCl and KCN/NaOH drops were mixed by tipping the plate, to release the HCN gas. Nematode paralytic killing was assessed as described above.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the on-line version, at doi:10.1016/j.micpath.2010.09.001.

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