Secondary Metabolites of *Pseudomonas fluorescens* CHA0 Drive Complex Non-Trophic Interactions with Bacterivorous Nematodes

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Received: 3 November 2010 / Accepted: 7 February 2011 / Published online: 1 March 2011
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Abstract Non-trophic interactions are increasingly recognised as a key parameter of predator–prey interactions. In soil, predation by bacterivorous nematodes is a major selective pressure shaping soil bacterial communities, and many bacteria have evolved defence mechanisms such as toxicity. In this study, we show that extracellular secondary metabolites produced by the model soil bacterium *Pseudomonas fluorescens* CHA0 function as a complex defence strategy against bacterivorous nematodes. Using a collection of functional mutants lacking genes for the biosynthesis of one or several extracellular metabolites, we evaluated the impact of bacterial secondary metabolites on the survival and chemotactic behaviour of the nematode *Caenorhabditis elegans*. Additionally, we followed up the stress status of the nematodes by measuring the activation of the abnormal DAuer Formation (DAF) stress cascade. All studied secondary metabolites contributed to the toxicity of the bacteria, with hydrogen cyanide efficiently repelling the nematodes, and both hydrogen cyanide and 2,4-DAPG functioning as nematicides. Moreover, these metabolites elicited the DAF stress response cascade of *C. elegans*, showing that they affect nematode physiology already at sublethal concentrations. The results suggest that bacterial secondary metabolites responsible for the suppression of plant pathogens strongly inhibit bacterivorous nematodes and thus likely contribute to the resistance of bacteria against predators in soil.

Introduction

Fluorescent pseudomonads are ubiquitous soil bacteria playing an important role in promoting plant health. Many strains produce secondary metabolites, such as 2,4-diacyethylphloroglucinol (DAPG), phenazines and hydrogen cyanide, thereby inhibiting soil-borne pathogens [1]. The success of these bacteria depends on various factors, such as their ability to efficiently exploit root exudates [2], but also to withstand predation by nematodes and protozoa [3, 4]. Predation is a major cause of bacterial mortality and profoundly impacts the structure and function of root-associated bacterial communities [5, 6]. Bacteria have evolved an array of antipredatory resistance mechanisms, such as toxicity, and in soil, unpalatable or toxic strains gain competitive advantage in presence of predators [3, 7]. Therefore, the production of secondary metabolites by biocontrol bacteria serves multiple functions, and metabolites protecting plants against pathogens improve for example bacterial resistance against predators [8, 9].

In this study, we investigated the role of secondary metabolites produced by the model soil bacterium *Pseudomonas fluorescens* CHA0 [10] interacting with the bacterivorous nematode *Caenorhabditis elegans*. Nematodes are major consumers of rhizosphere bacteria [5], and we hypothesised that bacterial secondary metabolites may inhibit and deter predators. Bacterial metabolites have been reported to affect nematode behaviour [11] and growth [12, 13]. Therefore, we tested if secondary metabolites produced by *P. fluorescens* function as chemotactic repellents and nematostatic compounds. At low concentrations, bacterial
metabolites can also stress nematodes, potentially reducing reproduction rate [14]. In nematodes, reproduction and longevity are controlled by the abnormal DAuer Formation (DAF) stress response cascade. This cascade triggers immune and detoxification mechanisms [14] and increases stress resistance, but at the cost of a reduced fertility [14]. We therefore chose this cascade as a proxy for the stress status of the nematodes.

We used a set of functional mutants of P. fluorescens CHA0 not producing one or several secondary metabolites to investigate the relative importance of each metabolite [9] for the defence against bacterivorous nematodes. We investigated three modes of action of the metabolites. Secondary metabolites may function as repellent, and we analysed the chemotactic response of nematodes to bacterial metabolites in a food choice experiment. Moreover, we tested the relative nematicide activity of the different metabolites in a slow killing assay with functional mutants of P. fluorescens CHA0. Furthermore, we monitored the potential of the secondary metabolites to stress the nematodes by using GFP fusions to follow the intracellular localisation of DAF-16, the negatively regulated transcription factor of the DAF-2 insulin-like signalling pathway of C. elegans, which controls the stress response of the nematodes to environmental stressors [15].

Materials and Methods

Organisms and Cultivation

Organisms used in this study are listed in Table 1. C. elegans was routinely kept at 14°C on NGM agar plates (peptone 2.5 g L⁻¹, NaCl 3 g L⁻¹, agar 17 g L⁻¹, K₂PO₄ 25 mM, MgSO₄ 1 mM, cholesterol 0.05 mg L⁻¹, CaCl₂ 1 mM) seeded with Escherichia coli OP50. P. fluorescens CHA0 and derivatives were grown on nutrient agar (blood agar base 40 g L⁻¹, yeast extract 5 g L⁻¹) with appropriate antibiotics (Table 1). Each of the tested knockout mutants lacks genes for the production of one secondary metabolite but still produces the others. The GacS⁻ mutant CHA19, which is deficient in cell signalling and does not produces any of the known secondary metabolites, was also included as reference non-toxic strain. Prior to experiments, a single colony of each tested strain was picked and incubated overnight at 28°C in liquid LB broth and washed twice in M9 buffer (KH₂PO₄ 3 g L⁻¹, Na₂HPO₄ 6 g L⁻¹, NaCl 5 g L⁻¹, MgSO₄ 250 mg L⁻¹).

Preference Test

In order to determine the potential of the different secondary metabolites as repellent against nematodes, we investigated the chemotactic response of C. elegans confronted with wild-type P. fluorescens CHA0 and isogenic mutants lacking genes for the production of one of the main secondary metabolites according to a standard food preference test [16] with few modifications. Ten microlitres of the tested strains (OD₆₀₀=0.5) were inoculated on one side of a 10-cm NGM agar plate. The other side of the plate was inoculated with the same amount of the wild-type reference strain P. fluorescens CHA0 producing secondary metabolites or the non-toxic strain CHA19. Additionally, two control treatments were set up with the CHA0 and CHA19 strains on both plate sides. For each treatment, five replicates were set up.

Nematodes (mixed developmental stages) were washed from the plates with M9 buffer, and 20 μL of the suspension was spotted in the middle of the plate at equal distance of two bacterial drops. Nematodes present at each side of the plate were counted after 24 h, and nematode preference for each strain against each of the two reference strains was calculated as fraction of total nematodes on the test side.

Slow Killing Assay

The impact of the different P. fluorescens strains on the fitness of nematodes was tested in a slow killing assay. Overnight cultures of the tested P. fluorescens strains were washed with M9 buffer and diluted to an OD₆₀₀ of 0.5. Fifteen microlitres of the bacteria were inoculated per well on 1/100 TSB agar (agar, 15 g L⁻¹, Tryptic Soy Broth 300 mg L⁻¹) in 24-well microtitre plates for 2 h (6 replicates each). Nematodes (mixed developmental stages) were washed with M9 buffer and added to the plates (10 μL per well, ca. 60 individuals). Dead and living nematodes were counted separately at the beginning of the experiment and after 24 h. Nematodes without detectable movement were judged as dead.

Stress Response of C. elegans—DAF-16 Translocation Assay

The influence of toxic P. fluorescens CHA0 and its isogenic mutants on the stress response of C. elegans was tested using a DAF-16 translocation assay [15]: GFP-labelled nematodes (strain CF1139) were picked from NGM agar plates previously inoculated with E. coli OP50, placed on M9 plates seeded previously with 50 μL P. fluorescens CHA0 or derivatives (see Table 1) at an OD₆₀₀ of 0.5, and incubated at 14°C for 12 h. Heat-stressed nematodes (2 h at 37°C) were used as positive control for DAF pathway activation [15].

In order to confirm the effect of KCN and DAPG on nematode stress status, worms were transferred to M9 agar...
plates supplemented with KCN (final concentration 80–5,000 μM) or DAPG (final concentration 8–500 μM) and incubated at 14°C for 12 h. The degree of nuclear translocation of DAF-16::GFP was characterised on the basis of the nuclear GFP fluorescence using three levels (no, weak, strong) corresponding to a cytoplasmic, intermediate and nuclear location of DAF-16::GFP, respectively [15]. The stress level of the nematodes was evaluated on 3×10 nematodes for each bacterial strain under an epifluorescence microscope by counting the proportion of nematodes showing either a weak or strong nuclear GFP fluorescence [15].

Statistical Analyses

The results were analysed by one-way analysis of variance investigating effects of the bacterial strains, followed by Tukey’s HSD test for comparison of means (α=0.05). Variables were checked for homogeneity of variances and percentages were arcsine square root transformed prior to the analyses. All analyses were performed using R 2.8.0. Error bars indicate standard error. Dose–response of the nematodes to KCN and DAPG was fitted with Michaelis–Menten kinetics to determine the toxin concentration required to induce half the maximum stress reaction. All experiments were repeated with similar results.

Results

Preference Test

Orientation of C. elegans towards the non-toxic P. fluorescens strain CHA19 as well as towards the wild-type strain CHA0 significantly varied with the P. fluorescens mutants not producing one of the main secondary metabolites offered as alternative food substrate (F_{5,20}=3.26, p=0.026 and F_{5,20}=16.37, p<0.001, respectively; Fig. 1). Offering the wild-type strain CHA0 as reference food source, nematodes preferred the mutants lacking genes for one or more secondary metabolites (Fig. 1A). However, the lack of single metabolites was usually not sufficient to modify significantly nematode behaviour. Only the GacS− strain was avoided most. The chemotactic behaviour was more pronounced offering the non-toxic strain CHA19 as reference food source; the nematodes preferred this strain to the other isogenic mutants with the exception of the strains not producing HCN and extracellular protease (Fig. 1B). Strains CHA1012 (PLT−) and CHA631 (DAPG−) were most avoided by the nematodes.

Slow Killing Assay

The slow killing assay confirmed that exoproducts of P. fluorescens function as nematode toxins (Fig. 2). The non-toxic strain CHA19 resulted in the lowest mortality, whereas mortality was at a maximum in the presence of strain CHA1012 (PLT+, DAPG++; F_{5,30}=29.91, p<0.001) suggesting DAPG as potent toxin killing C. elegans. The fraction of nematodes killed when exposed to CHA0, CHA207 and CHA631 was intermediate. Knocking out single metabolite genes was not sufficient to eliminate the nematicide activity, suggesting that all tested exoproducts contributed to bacterial toxicity and/or that some of them do not kill nematodes.

Stress Response of C. elegans

Incubation of nematodes with the wild-type strain P. fluorescens CHA0 resulted in nuclear translocation of DAF-16 in about 65% of the worms after 12 h (Fig. 3). Incubation with isogenic mutants lacking genes for the biosynthesis of one of the secondary metabolites resulted

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**Table 1** Organisms used in this study

| Strain         | Characteristics                                      | Reference |
|----------------|------------------------------------------------------|-----------|
| *P. fluorescens* | Wild type; DAPG− PLT+ PRN+ HCN+ AprA+               | [34]      |
| CHA0           | ΔgacS; DAPG− PLT− PRN− HCN− AprA−                   | [35]      |
| CHA207         | hcnA;lacZ; DAPG− PLT− PRN− HCN− AprA−               | [36]      |
| CHA631         | ΔphlA DAPG− PLT++ PRN+ HCN+ AprA+                   | [22]      |
| CHA805         | AprA−; lacZ; DAPG− PLT− PRN− HCN− AprA−             | [27]      |
| CHA1012        | pltB::Tn5 Km+; DAPG− PLT− PRN− HCN− AprA−           | [28]      |
| E. coli        | Standard laboratory strain                           |           |
| OP50           | Wild type                                            |           |
| CF1139         | DAF-16::GFP (mu86) I; muls61                         | [15]      |

+ toxin production, − lack of the toxin, ++ toxin overproduction compared to wild-type strain, DAPG 2,4-diacetylphloroglucinol, PLT pyoluteorin, PRN pyrrolnitrin, HCN hydrogen cyanide, AprA extracellular protease

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in a lower percentage of worms showing DAF-16 nuclear translocation ($F_{5,12}=14.20$, $p<0.001$; Fig. 3), and the GacS$^-$ mutant did not elicit translocation at all, suggesting that the DAF cascade is activated by the secondary metabolites of \textit{P. fluorescens}. In particular, mutants not producing DAPG and, to some extent, mutants not producing cyanide reduced the nuclear translocation of DAF-16 (Fig. 3), suggesting that these metabolites are main elicitors of the DAF stress response pathway. Pure DAPG and KCN elicited nuclear translocation of DAF-16 with a half saturation constant of 30 and 130 $\mu$M for DAPG and KCN, respectively, confirming their effect as stressors. Notably, KCN did not stress the worms, even at 5 mM only 80% of the worms showed nuclear translocation.

Figure 1 Chemotactic preference of \textit{C. elegans} for different mutants of \textit{P. fluorescens} CHA0 after 24 h. Nematodes were offered wild-type cells or isogenic mutants deficient in the production of pyoluteorin (PLT$^-$, CHA1012), 2,4-diacetylphloroglucinol (DAPG$^-$, CHA631), extracellular proteases (AprA$^-$, CHA805), hydrogen cyanide (HCN$^-$, CHA207) or all extracellular metabolites (GacS$^-$, CHA19). Nematode preference was assessed against the wild-type, toxic strain CHA0 (a) or the non-toxic strain CHA19 (b). Error bars indicate $\pm$SE; different letters indicate significant differences between means at $p<0.05$ (Tukey’s HSD test).

Figure 2 Toxicity of different mutants of \textit{P. fluorescens} CHA0 against \textit{C. elegans} in the slow killing assay after 28 h. Nematodes were incubated with the wild-type strain or isogenic mutants deficient in the production of pyoluteorin (PLT$^-$, CHA1012), 2,4-diacetylphloroglucinol (DAPG$^-$, CHA631), extracellular proteases (AprA$^-$, CHA805), hydrogen cyanide (HCN$^-$, CHA207) or all secondary metabolites (GacS$^-$, CHA19). Error bars indicate $\pm$SE; different letters indicate significant differences between means at $p<0.05$ (Tukey’s HSD test).

Figure 3 Effect of \textit{P. fluorescens} CHA0 and its isogenic mutants on the activation of the DAF stress response of \textit{C. elegans}. Stress response was assessed by following nuclear translocation of the DAF-16::GFP fusion (a). Nematodes were incubated at 14°C on M9 plates overlayed with \textit{P. fluorescens} strains CHA0 (WT), CHA19 (GacS$^-$), CHA207 (HCN$^-$), CHA805 (AprA$^-$), CHA1012 (PLT$^-$) and CHA631 (DAPG$^-$) for 12 h. Individuals showing translocation were counted as stressed (b). Error bars indicate $\pm$SE; different letters indicate significant differences between means at $p<0.05$ (Tukey’s HSD test).
Discussion

Secondary metabolites form a key component of the defence response of soil pseudomonads against bacterivorous nematodes [4, 17], and likely contribute to improve bacterial fitness in soil. We showed that extracellular metabolites of \textit{P. fluorescens} CHA0 drive complex interactions with nematode predators, affecting both nematode physiology and behaviour. Interestingly, the different secondary compounds differentially affected nematodes, acting as repellents, stressors or nematicides. Compared to the wild type, the effect of most mutants on nematodes was reduced. However, no single knockout was sufficient to entirely suppress bacterial toxicity suggesting that various secondary metabolites are involved in the interaction with nematodes. Mutants deficient in the production of one secondary metabolite still produce the remaining ones, and one has to consider that knocking out one compound may result in increased expression of another. In this study, we tested the role of four important and well-described secondary metabolites in the interaction with nematodes. Genome analyses of the closely related strain \textit{P. fluorescens} Pf-5 suggest that more secondary metabolites are produced [18], although little information is yet available. In addition, \textit{P. fluorescens} Pf-5 possesses a type VI secretion system [19], indicating that other mechanisms than extracellular secondary metabolites contribute to the interaction with nematodes.

Nematodes showed a strong negative chemotactic behaviour against the toxic wild-type and markedly preferred the toxin-deficient \textit{P. fluorescens} strain CHA19, suggesting that secondary metabolites help to repel nematodes. Many bacteria produce defence toxins, and avoidance of toxic prey may be crucial for the survival of nematodes [20], which use a highly developed sensory system to detect toxic prey [21]. Avoidance of mutants suggests that the production of HCN by \textit{P. fluorescens} plays a central role in deterring nematodes. In fact, HCN is a potent inhibitor of respiratory pathways and causes paralytic death of nematodes [12]. Interestingly, nematodes preferred the wild-type strain to the mutant lacking 2,4-DAPG. Knocking out the production of 2,4-DAPG results in an overproduction of pyoluteorin (PLT) and vice versa [22]. The avoidance of the DAPG deficient (and PLT overproducing) strain therefore suggests that at high concentration, this compound may function as repellent. The avoidance was even more pronounced when tested against the non-toxic GacS\(^{-}\) strain. In this case, PLT- and DAPG-deficient mutants were the most avoided, suggesting that single overproduced secondary metabolites strongly affect the chemotactic behaviour of nematodes. However, since both mutants still produce HCN, the effect of the tested exoproduct may have been masked. Construction of multiple knockout mutants of \textit{P. fluorescens} producing only one single secondary metabolite may help to better understand the contrasting effect of individual secondary metabolites on nematode behaviour.

Knockout mutants deficient in the production of one of the secondary metabolites showed an altered toxicity against the nematodes in the slow killing assay. With the exception of the GacS\(^{-}\) strain (lacking all exoproducts), the strain deficient in HCN production showed the lowest toxicity. Indeed, as stated above, HCN is known to kill effectively nematodes [12] and presumably is one of the most effective nematicides produced by \textit{P. fluorescens} CHA0 [23]. Mutants deficient in DAPG synthesis did not significantly differ from the wild type, but interestingly, the most toxic strain was the PLT\(^{-}\) strain, lacking pyoluteorin and overproducing DAPG [22]. This suggests that in addition to HCN, DAPG may act as nematicide at high concentration. By inhibiting mitochondrial activity [24], DAPG plays an important role in the inhibition of competing bacteria [10] and predators [9, 25], but also in the control of plant-parasitic nematodes by pseudomonads [13, 26]. Extracellular proteases are another class of compounds functioning as nematicide, and in agreement with previous studies on root knot nematodes [27], deactivation of the production of the alkaline protease AprA resulted in reduced toxicity of bacteria against \textit{C. elegans}. Surprisingly, the PLT-deficient mutant of \textit{P. fluorescens} was more toxic for \textit{C. elegans} than the wild type, while the DAPG-deficient mutant presented reduced toxicity. Production of DAPG and PLT is balanced, each compound inhibiting the synthesis of the other, and PLT-deficient mutants overproduce DAPG [28]. Consequently, the higher toxicity of the PLT-deficient mutant probably was an indirect effect of DAPG overproduction, underlining the toxicity of DAPG.

In addition to nematicidal effects, bacterial secondary metabolites influenced the physiology of the nematodes. \textit{P. fluorescens} CHA0, but not its non-toxic GacS\(^{-}\) mutant, induced strong nuclear translocation of the DAF-16 transcription factor, indicating stress response of the nematodes after exposure to bacterial exoproducts. Nematodes possess an extensive innate immune system, and \textit{C. elegans} uses conserved cellular signalling pathways to detect and respond to pathogens [29]. The DAF-2 stress response cascade is activated by a number of stress factors including bacterial toxins [30], and controls the investment in defence and reproduction [31, 32]. In contrast to pathogens, which circumvent the immune response of nematodes preventing DAF-16 translocation [33], soil bacteria may benefit from activating the nematode stress response. Since the DAF cascade also regulates nematode reproduction, sublethal levels of bacterial toxins likely reduce predator fitness in the long term. Mutants not...
producing DAPG induced the lowest nuclear translocation, and tests with pure DAPG confirmed that nematodes were stressed at very low DAPG concentration, confirming the central role of this toxin in the interaction between P. fluorescens and nematodes. More detailed analyses of the expression of the genes downstream of the DAF cascade in the presence of bacterial toxins are needed to understand the molecular mechanisms allowing nematodes to withstand toxic bacteria in soil.

Acknowledgements We are grateful to Tim Janowitz and Peter Gerke (University of Münster) and Susanne Böning-Klein (University of Göttingen) for their assistance in the experiment on the stress response of C. elegans. AJ was funded by the fellowship programme of the German Federal Foundation for the Environment (DBU).

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