Pseudomonas fluorescens CHA0 can kill subterranean termite Odontotermes obesus by inhibiting cytochrome c oxidase of the termite respiratory chain

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Received 18 June 2009; accepted 24 August 2009. Final version published online 23 September 2009.
DOI:10.1111/j.1574-6968.2009.01782.x

Editor: Juan Imperial

Keywords
Pseudomonas fluorescens CHA0; Odontotermes obesus; cytochrome c oxidase; termite; hydrogen cyanide.

Abstract

Pseudomonas fluorescens CHA0 has been shown to suppress the growth of a wide range of microbial plant pathogens as well as invertebrate pests such as root nematodes. Hydrogen cyanide, a secondary metabolite produced by the bacterium, has been credited as being one of the determinants of its biocontrol ability. The use of biocontrol agents against social insect pests such as termite Odontotermes obesus has limitations because of behavioural adaptations that include (1) removal of the pathogen when grooming by the termites and (2) isolation of infested members of the colony. In this study, we show that cyanide of bacterial origin may inhibit cytochrome c oxidase (CCO) of the termite respiratory chain and demonstrate that HCN-producing bacteria such as P. fluorescens can actually kill a macroscopic insect pest by cyanide poisoning. This ability of pseudomonal metabolites such as cyanide, which can bring about pest death by blocking respiration through inhibition of CCO rather than infection or predation, can potentially overcome the behavioural adaptations of social insect pests such as termites and represents an attractive option for insect pest management.

Introduction

Pseudomonas fluorescens CHA0 is a gram-negative bacterium that can inhibit the growth of a wide range of organisms, including bacteria, plants and nematodes. Pseudomonas fluorescens CHA0 produces hydrogen cyanide (HCN), one of the metabolites believed to be responsible for the biocontrol ability of this strain (Blumer & Haas, 2000; Ramette et al., 2003). HCN is a typical pseudomonal secondary metabolite, a compound that is not required for growth, energy storage or primary metabolism, but that may confer some ecological advantage to the organism (Vining, 1990; Gallagher & Manoil, 2001). Cyanide is a potent toxin that causes death in vertebrates by inactivating cytochrome oxidase (Ikegaya et al., 2001). Gallagher & Manoil (2001) had suggested that a similar inhibition of cytochrome oxidase could be a mechanism of growth suppression in nematodes by HCN-producing Pseudomonas aeruginosa PA01, but did not provide experimental proof. Here we demonstrate that P. fluorescens CHA0 may indeed cause death in Odontotermes obesus (Rambur) – an important termite pest of the Indian subcontinent that causes extensive damage to major agricultural crops and forest plantation trees (Rajagopal, 2002; Kakde et al., 2005) through inactivation of cytochrome c oxidase (CCO) in the termite respiratory electron transport chain.

While the ability of HCN-producing bacteria to kill microbial and invertebrate pests has been shown previously (Gallagher & Manoil, 2001; Siddiqui et al., 2006; Devi et al., 2007), to the best of our knowledge, the present study is the first to demonstrate that HCN-producing bacteria such as P. fluorescens CHA0 may inhibit CCO – a key component of the respiratory electron transport chain.

Materials and methods

Odontotermes obesus was collected from the Bhatti Wildlife sanctuary, New Delhi. Nest fragments were transported to the laboratory in polypropylene containers, and experiments were performed within 2–4 days after
collection. Worker caste termites were used for all the experiments.

**Bacterial strains**

Wild-type, HCN-producing *P. fluorescens* CHA0 strain and a Δ*hcnc* mutant *P. fluorescens* CHA77 were used in the present study. Strain CHA77 was obtained by creating a 2.4 kb PstI deletion within the *hcncABC* genes and transferring to the chromosome of strain CHA0 using the double-crossover technique (Laville et al., 1998). Strain CHA77 is therefore identical to CHA0 in all characteristics, except that it does not produce HCN.

We have previously demonstrated that HCN production in HCN-producing bacteria varies with time (Devi et al., 2007). In order to expose termite *O. obesus* to different concentrations of HCN, we used 6-, 12-, 18- and 24-h-old bacterial cultures grown in nutrient broth (pH 7.0) at 30 °C. The number of bacterial cells per microlitre of culture was estimated following Benson (1990). HCN production in the two strains was estimated following Devi et al. (2007). The sensitivity of the method is 6 μM HCN.

**Termiticidal effects of *P. fluorescens***

One hundred and fifty termites were taken in each Petri dish containing a Whatman paper no. 1 fixed to its underside. After moistening the Whatman paper with 50 mL of 6-, 12-, 18- and 24-h-old bacterial culture suspensions, the Petri dishes were covered and left for 2 h. A Petri dish with termites to which sterile nutrient medium was added served as the control. For direct exposure to exogenous cyanide, the termites were taken in a Petri dish containing a Whatman paper no. 1 fixed to its underside. After moistening the Whatman paper with 50 mL sterile nutrient medium supplemented with 0, 5, 15, 20, 30, 45 and 60 μM KCN, the Petri dishes were covered and left for 2 h. After a 2-h treatment, the termites were rated as alive or dead. The termites were considered dead if they did not move spontaneously and did not respond detectably to tapping of the Petri dish. Three replicates were maintained for each treatment.

**Termite CCO activity**

Following a 2-h incubation with the different treatments, liquid nitrogen was poured into the plates to kill any termites that remained alive after the period of incubation. In order to confirm that the cause of inhibition in CCO activity was exposure to the treatment provided and not death of the termites, Petri plates were set up (three replicates) with a sterile medium in which all the termites were first killed with liquid nitrogen and allowed to stand for 2 h before assaying for CCO activity.

Termite mitochondria were prepared by differential centrifugation of termite tissue homogenates following Ikegaya et al. (2001). Termites incubated with (1) sterile nutrient broth; (2) 6-, 12-, 18- and 24-h cultures of wild-type, HCN-producing *P. fluorescens* CHA0 in nutrient broth; (3) 6-, 12-, 18- and 24-h cultures of *P. fluorescens* CHA77 (Δ*hcnc* mutant) that did not produce HCN; and (4) sterile nutrient broth supplemented with 0–60 μM KCN were placed in buffer (10 mM Tris, 0.2 mM EDTA, 0.25 M sucrose, pH 7.6) and homogenized. The homogenate was centrifuged at 1500 g for 10 min and the pellet was discarded. The supernatant was centrifuged at 8000 g and the new supernatant was discarded. The pellet was resuspended in the same buffer and centrifuged at 8000 g again. The new pellet was resuspended in buffer (10 mM Tris, 0.2 mM EDTA, pH 7.6) at 1 mg protein mL⁻¹.

Cytochrome *c* reaction mixture (63 μM cytochrome *c*, 10 mM Tris, 0.2 mM EDTA, 0.05% *n*-dodecyl-β-D-maltoside, pH 7.6) was incubated at 25 °C for 30 min with 12.5 μM L(-)-ascorbic acid, to convert ferric cytochrome *c* to ferrous cytochrome *c*. Thirty microlitres of the 1 mg mL⁻¹ termite mitochondrial protein solution was then added to 3 mL of the reaction mixture (Ikegaya et al., 2001). The oxidation of ferrous cytochrome *c* by CCO was spectrophotometrically monitored for 2 min at 1-min intervals. A change in absorbance between 0 and 1 min indicated CCO activity (Ikegaya et al., 2001). The specific enzyme activity of CCO was calculated using the molar extinction coefficient of ferrous cytochrome *c* (0.79 μmol mL⁻¹ cm⁻¹).

**Results**

**Termiticidal effects of *P. fluorescens***

The number of bacterial cells produced, the amounts of HCN released and the mortality caused in termites by HCN production by strain CHA0 increased from nil (after 6 h of growth) to 20% after 24 h of growth in nutrient medium are presented in Table 1. Twenty-four-hour cultures of CHA0 consistently killed all the termites after a 2-h incubation period. HCN production by strain CHA0 from nil (after 6 h of growth) to 20% (after 24 h of growth). The increase in HCN production with time resulted in increased mortality in the termites (Table 1) that was positively correlated ($R^2 = 0.85$) with the concentration of HCN released by the bacterial cells. A similar correlation ($R^2 = 0.80$) was found between per cent mortality and cyanide concentration in termites incubated with exogenous KCN (Fig. 1). While no mortality was found in termites incubated with the control and 6–18-h-old cultures of strain CHA77, 30% mortality was found in termites incubated with 24-h-old cultures of CHA77 that did not produce measurable amounts of HCN.
and 2012-, 18- and 24-h cultures of CHA0, which released 14, 16 protein and CCO activities in termites incubated with FEMS Microbiol Lett. Per cent mortality in Fig. 1. Growth, HCN production and per cent mortality caused in termites by 6-, 12-, 18- and 24-h-old cultures of wild-type HCN-producing Table 1. Termite CCO activity needs further investigation. P. fluorescens CHA0 kills O. obesus by inhibiting CCO

**Termite CCO activity**

The specific enzyme activity of CCO in worker caste termites of O. obesus incubated with a sterile nutrient medium (control) was $5.51 \pm 0.38 \text{ U mg}^{-1}$ protein. CCO activity in termites incubated with the HCN-producing bacteria was dependent on the amounts of HCN produced in culture (Fig. 2). For instance, the specific enzyme activity of CCO in termites incubated with 6-h culture of wild-type CHA0 and 6–18-h cultures of Δhcn mutant CHA77 that did not produce HCN were not significantly different from the CCO activity of termites exposed to a sterile nutrient medium (Fig. 2), while CCO activity in termites incubated with 12–24-h cultures of CHA0 that produced HCN was significantly lower (Fig. 2). Interestingly, a 16% reduction in CCO activity was also found in termites incubated in a 24-h culture of CHA77. The inhibition in CCO activity caused by 12–24-h cultures of CHA0, which produced HCN, was dependent on the amounts of HCN produced and similar to inhibition caused in termites incubated with exogenous KCN. For instance, CCO activities in termites exposed to 15 and 20 µM KCN were 3.52 ± 0.40 and 3.18 ± 0.12 U mg$^{-1}$ -protein and CCO activities in termites incubated with 12-, 18- and 24-h cultures of CHA0, which released 14, 16 and 20 µM HCN, were 3.86 ± 0.19, 3.18 ± 0.34 and 2.80 ± 0.12 U mg$^{-1}$ protein, respectively. Termites incubated with 0–60 µM exogenous KCN demonstrated that CCO inhibition was indeed dependent on and inversely correlated ($R^2 = 0.90$) with cyanide concentration (Fig. 3).

**Discussion**

Siddiqui et al. (2006) have earlier demonstrated the ability of the CHA0 strain of P. fluorescens to suppress the growth of root-knot nematode, Meloidogyne javanica, by cyanide poisoning. In the present study, a 60 µM concentration of exogenously supplied KCN killed all the termites (Fig. 1), whereas 24-h-old cultures of P. fluorescens strain CHA0 consistently killed all termites with much lower concentrations of 20 µM HCN (Table 1) under similar conditions. It is difficult to explain this difference in mortality with the available data. Pseudomonas fluorescens CHA0 is known to produce several active metabolites in addition to HCN (Voissard et al., 1994; Troxler et al., 1997; Haas & Défago, 2005; Jouset et al., 2006). This could be a reason for the differences in mortality observed. Thirty per cent mortality was also found in termites incubated with the Δhcn mutant strain CHA77 that did not produce measurable amounts of HCN. This indicates that other metabolites may indeed play a role in causing termite death. However, the fact that no mortality was found in termites exposed to a 6-h culture of CHA0 that did not produce HCN and 6–18-h cultures of CHA77 suggests that bacterial HCN may be a primary cause

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Bacteria mL$^{-1}$</th>
<th>HCN production (µM)</th>
<th>Termite mortality (%)</th>
<th>Bacteria mL$^{-1}$</th>
<th>HCN production (µM)</th>
<th>Termite mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>$3.50 \pm 0.50 \times 10^7$</td>
<td>&lt; 6</td>
<td>0</td>
<td>$2.80 \pm 0.20 \times 10^7$</td>
<td>&lt; 6</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>$3.0 \pm 0.50 \times 10^8$</td>
<td>14 ± 2</td>
<td>79.0 ± 3.60</td>
<td>$3.30 \pm 0.20 \times 10^8$</td>
<td>&lt; 6</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>$4.60 \pm 0.50 \times 10^8$</td>
<td>16 ± 1</td>
<td>92.66 ± 2.51</td>
<td>$4.20 \pm 0.20 \times 10^8$</td>
<td>&lt; 6</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>$5.20 \pm 0.80 \times 10^8$</td>
<td>20 ± 3</td>
<td>All dead</td>
<td>$4.30 \pm 0.75 \times 10^8$</td>
<td>&lt; 6</td>
<td>30.0 ± 0</td>
</tr>
</tbody>
</table>

Values represent average of three replicates ± SD of the mean.

**Fig. 1.** Per cent mortality in Odontotermes obesus worker caste termites incubated for 2 h with exogenously supplied KCN (0–60 µM). Values represent average of three replicates and the vertical bars indicate SD of the mean.

P. fluorescens CHA0 kills O. obesus by inhibiting CCO
for mortality in the termites incubated with 12–24-h cultures of CHA0.

The percent mortality in termites incubated with exogenous KCN was correlated \((R^2 = 0.89)\) with the inhibition of CCO activity in the termites. Gallagher & Manoil (2001) had suggested that inhibition of cytochrome oxidase could be a mechanism of growth suppression in the nematode Caenorhabditis elegans by HCN-producing P. aeruginosa PAO1. CCO is the terminal catalyst in the mitochondrial respiratory chain and is involved in electron transport and proton translocation across the membrane (Saraste, 1990; Gennis, 1992). Any substance that inactivates CCO can, therefore, block the respiratory chain. Cyanide and carbon monoxide (CO), in particular, are potent toxins that inactivate CCO in mammalians (Ikegaya et al., 2001). Cyanide causes a rapid onset of anoxia by inhibiting mitochondrial oxidative phosphorylation through binding to the CCO heme \(a_3-Cu_B\) binuclear centre to inhibit both cellular oxygen utilization and ATP production (Leavesley et al., 2008).

However, cyanide production in bacteria is tightly regulated, with local concentrations usually being \(< 1\) mM, and can be tolerated by many living cells. Consequently, reports that describe acute or chronic toxicity caused by bacterial cyanide in animals or plants are rare (Blumer & Haas, 2000).

Inhibition of CCO activity (Fig. 2) and consequent death in termite O. obesus caused by HCN-producing P. fluorescens were dependent upon the amounts of HCN produced. Termites incubated with exogenous KCN confirmed that CCO inhibition was dependent on and inversely correlated with cyanide concentration (Fig. 3). However, a 16% reduction in CCO activity was also found in termites incubated with strain CHA77, which did not produce HCN. Strain CHA77 produces all metabolites that strain CHA0 produces, except HCN. Pseudomonad metabolites such as phenazine and pyrrolnitrin have been reported to inhibit electron transport (Haas & Défago, 2005) in different organisms. This could be a cause for the observed reduction in CCO activity in termites incubated with strain CHA77. This needs further investigation. The CCO activity in termites that were killed with liquid nitrogen and allowed to stand for 2 h before assay (of CCO activity) was 5.43 ± 0.53 U mg\(^{-1}\) protein. This is not significantly different from CCO activity of

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**Fig. 2.** Specific enzyme activity of CCO in Odontotermes obesus worker caste termites incubated for 2 h with a sterile nutrient medium (control) and 6-, 12-, 18- and 24-h-old cultures of wild-type HCN-producing Pseudomonas fluorescens CHA0 and P. fluorescens CHA77 (Δhcn mutant). Values represent the average of three replicates, and the vertical bars indicate the SD of the mean.

**Fig. 3.** Correlation between exogenous KCN and specific enzyme activity of CCO in Odontotermes obesus worker caste termites exposed to 0–60 \(\mu\)M of KCN for 2 h. Values represent the average of three replicates, and the vertical bars indicate SD of the mean \((R^2 = 0.90)\).
termites incubated with a sterile nutrient medium (5.51 ± 0.38 U mg⁻¹ protein). This indicates that death does not cause a significant inhibition in CCO activity (for up to 2 h) and that the reduction in CCO activity observed in termites incubated with various treatments may be an outcome of exposure to cyanide and other bacterial metabolites.

Although 100% mortality was found in termites exposed to 24-h cultures of CHA0 and 60 μM exogenous KCN, there was a difference in the CCO activity. The CCO activity in termites incubated with 24-h cultures (which released 20 μM HCN) was higher (2.80 ± 0.12 U mg⁻¹ protein) than in termites incubated with 60 μM KCN in which the CCO activity was 1.57 ± 0.19 U mg⁻¹ protein. This finding indicates that cyanide (exogenous or of bacterial origin) may play a role in inhibiting CCO activity in termites. This is further evidenced by findings that show that, although mortalities in termites incubated with 12-, 18- and 24-h cultures of CHA0, which released 14, 16 and 20 μM HCN, respectively, were higher than in termites incubated with 15 and 20 μM KCN, the CCO activities were similar. Nevertheless, the inhibition of CCO activity in termites incubated with the mutant CHA77 suggests that metabolites other than bacterial HCN may also be involved in inhibiting termite CCO activity.

Our study has shown that HCN-producing P. fluorescens can kill termites and may have the potential to protect plants from termite infestation. HCN-producing bacteria may offer an attractive biocontrol option against termite pests such as O. obesus, because strategies using natural predators and parasites in the management of termite infestation are often frustrated due to behavioural adaptations of termites that include (1) removal of the pathogen when grooming by the termites and (2) isolation of infested members of the colony (Wright et al., 2005). Because rhizospheric HCN-producing bacteria such as P. fluorescens are able to kill termites without actual infection or physical contact, their introduction into subterranean termite colonies could provide an effective means for containing the damage caused by termite species such as O. obesus. However, as soil conditions are more complex than those in vitro, additional studies on cyanogenesis and the production of other volatile metabolites by HCN-producing bacteria are required to develop this approach as a feasible and environmentally sound biological termite management method (Kremer & Souissi, 2001).

Acknowledgements

This study was facilitated by a research grant from the University of Delhi to D.K. The authors thank Prof. G. Défago and Dr M. Frapolli (Swiss Federal Institute of Technology, Switzerland) for strain CHA0; Prof. Dieter Haas and Laurene Rochat (University of Lausanne, Switzerland) for strain CHA77 (Ahcn mutant); Dr V.V. Ramamurthy (IARI, India) and Dr Mukhtar Ahmed (FRI, India) for identification of O. obesus; Dr Alban Ramette, Max-Plank Institute for Marine Biology, and Dr D.S. Rawat and Mr Mukul, Department of Chemistry, University of Delhi, for advice on HCN quantification; Dr R.S. Sharma and Prof. Inderjit, CEMDE for laboratory facilities; and the editor and two anonymous referees for comments that improved this manuscript.

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