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Extracellular Protease of *Pseudomonas fluorescens* CHA0, a Biocontrol Factor with Activity against the Root-Knot Nematode *Meloidogyne incognita*

Imran Ali Siddiqui,[1] Dieter Haas,[2] and Stephan Heeb[2]*

Soil Biology and Ecology Laboratory, Department of Botany, University of Karachi, Karachi-75270, Pakistan,[1] and Département de Microbiologie Fondamentale, Université de Lausanne, CH-1015 Lausanne, Switzerland[2]

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In *Pseudomonas fluorescens* CHA0, mutation of the GacA-controlled *aprA* gene (encoding the major extracellular protease) or the *gacA* regulatory gene resulted in reduced biocontrol activity against the root-knot nematode *Meloidogyne incognita* during tomato and soybean infection. Culture supernatants of strain CHA0 inhibited egg hatching and induced mortality of *M. incognita* juveniles more strongly than did supernatants of *aprA* and *gacA* mutants, suggesting that AprA protease contributes to biocontrol.

Plant diseases caused by soilborne root pathogens account for major crop losses worldwide. Yet in a small number of environments, i.e., in suppressive soils, little or no disease is observed, despite the presence of pathogens. Disease suppression depends, in part, on microorganisms that are able to antagonize pathogens (5, 10, 14, 28). The root-colonizing bacterium *Pseudomonas fluorescens* CHA0, which was isolated from a suppressive soil, has been studied in detail as a model strain for the biological control of several fungal plant diseases, such as black root rot of tobacco and take-all disease of wheat (5, 27). In this strain, as well as in other biocontrol pseudomonads, antifungal secondary metabolites, e.g., 2,4-diacetylphloroglucinol, hydrogen cyanide, and pyoluteorin, are important for biocontrol activity. These biocontrol factors are synthesized in response to environmental conditions and to population densities of the producer strain, whereby the GacS/GacA two-component system exerts a crucial role as a positive control element (6, 8, 9, 11, 26). Some rhizosphere microorganisms, including *P. fluorescens* CHA0, can also act as antagonists of plant-pathogenic nematodes (23). For antagonistic fungi, this biological control has been shown to involve extracellular proteases (2, 21). In strain CHA0, the production of the major extracellular EDTA-sensitive protease, AprA, is controlled by the GacS/GacA signal transduction pathway (8, 17, 26, 29). The present study was undertaken to find out whether this enzyme contributes to the biocontrol properties of strain CHA0 in plant-nematode interactions.

Characterization of the *aprA-aprI-aprD* gene region involved in production of the major exoprotease of strain CHA0. Strain CHA803, a Tn*5* insertion mutant derivative of wild-type CHA0 (20), lacked proteolytic and lipolytic activities on indicator agar plates (17, 18) but showed wild-type production of antifungal metabolites, indicating that the Tn*5* insertion was not in *gacS* or *gacA* (9). The Tn*5* insertion was mapped to the 3′ end of the

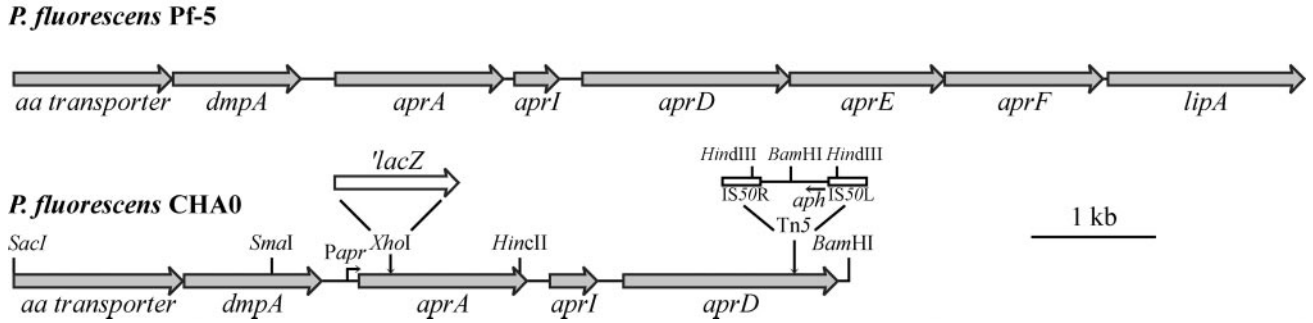

FIG. 1. Genetic organization of the region surrounding *aprA* in *P. fluorescens* CHA0 and Pf-5. The 6.7-kb SacI-BamHI fragment of strain CHA0, which was sequenced in this study (GenBank accession no. AY644718), is aligned with the homologous region of strain Pf-5 (http://www.tigr.org) shown above. The sites where a translational *'lacZ* fusion and a Tn*5* element are inserted in the chromosome of strains CHA805 and CHA803, respectively, are shown above the *aprA* and *aprD* genes. P*apr*, promoter of *aprA*; *aph*, kanamycin resistance gene.

* Corresponding author. Present address: Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom. Phone: 44 (0115) 951 50 89. Fax: 44 (0115) 846 79 51. E-mail: Stephan.Heeb@nottingham.ac.uk.

TABLE 1. *P. fluorescens* strains and plasmids

Strain or plasmid	Description	Reference
Strains of *P. fluorescens*		
CHA0	Wild type	27
CHA19	Δ*gacS*	29
CHA89	*gacA*::Km^r	11
CHA803	*aprD*::Tn*5*	20
CHA805	*aprA*::*'lacZ*	This study
CHA806	Δ*gacS aprA*::*'lacZ*	This study
Plasmids		
pME3087	Suicide vector; ColE1 replicon, IncP-1-Mob, Tc^r	27
pME6063	5.0-kb *aprA'-'lacZ-'aprA* fragment inserted into pME3087, suicide plasmid for *aprA*::*'lacZ* chromosomal fusions	This study

TABLE 2. In vitro effects of culture filtrates of *P. fluorescens* strains on *M. incognita* egg hatching and juvenile mortality

Strain or LSD[a]	Hatching[b]	Mortality[b]
Control	83a	18a
CHA0	51b	56b
CHA89	77a	25a
CHA805	70a	31a
LSD[c]	17	14

[a] Strains CHA0 (wild type), CHA89 (*gacA*), and CHA805 (*aprA'-'lacZ*) were grown in 1/20 King's B medium for 48 h. The control consisted of sterile medium.
[b] Hatching and mortality were tested as previously described (22) and are expressed as the percentage of eggs hatched after 72 h of exposure and as the percentage of juvenile mortality after 24 h of exposure, respectively. Data for each strain and medium were obtained from three different experiments with six repetitions. Means of the experiments in each column followed by different letters are significantly different ($P \leq 0.05$) according to Fisher's test.
[c] LSD, least significant difference.

aprD gene (Fig. 1), whose deduced amino acid sequence has 56% identity with the ATP-driven translocator AprD, a component of the type I secretion machinery required for the secretion of alkaline protease AprA in *P. aeruginosa* (1, 3). By a chromosome walking approach (7), the genes located upstream of *aprD*, that is, an open reading frame coding for an amino acid transporter, *dmpA* (for a putative aminopeptidase), *aprA* (for extracellular protease), and *aprI* (for the cognate protease inhibitor), were cloned and sequenced in strain CHA0 (Fig. 1). The genomic sequence of *P. fluorescens* Pf-5, which is phenotypically and genotypically very similar to *P. fluorescens* CHA0 (4, 15), predicts that the *aprAID* genes are the proximal part of an *aprAIDEF* operon, which includes the *lipA* gene (for extracellular lipase) at the 3′ end (Fig. 1).

The deduced *aprA* gene product shows 62% identity with the AprA alkaline protease of *P. aeruginosa* (3) and contains Zn^{2+}- and Ca^{2+}-binding motifs. The calculated molecular mass of 49.9 kDa for the secreted form of AprA is in reasonable agreement with the value (47.1 kDa) previously determined for the EDTA-sensitive, major exoprotease of strain CHA0 (17). Between the *aprA* and *aprD* genes lies the *aprI* gene (Fig. 1) coding for a predicted 13.8-kDa protein which shows 40% amino acid sequence identity with the *P. aeruginosa* AprI protein, an AprA-specific inhibitor (3).

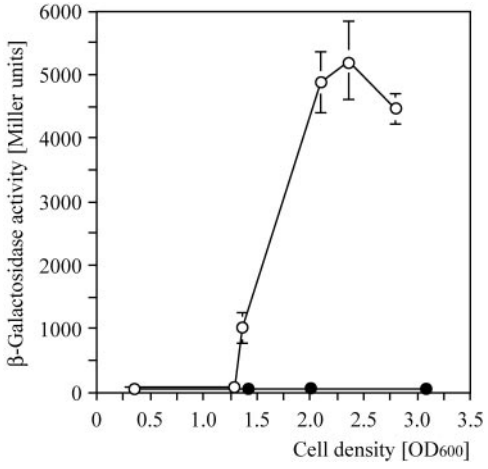

FIG. 2. GacS control of an *aprA*::*'lacZ* fusion in *P. fluorescens* grown in liquid King's B medium. β-Galactosidase activities were determined by the Miller method (13) for *aprA*::*'lacZ* in the wild-type derivative CHA805 (○) and in the *gacS* mutant CHA806 (●). The growth rates of both strains were similar (data not shown). Each value is the average ± standard deviation from three different cultures.

A nonpolar *aprA* mutation was constructed by the insertion of a *'lacZ* cassette into the unique XhoI site of the chromosomal *aprA* gene (Fig. 1) in the wild type and in a *gacS* background, using the suicide plasmid pME6063 (Table 1). This resulted in strains CHA805 and CHA806 (Table 1), respectively. Strain CHA805 was exoprotease negative, as expected, but lipase positive, in keeping with the nonpolar nature of the *'lacZ* insertion. β-Galactosidase activities of the *aprA'-'lacZ* translational fusion in strain CHA805 showed a marked cell density-dependent expression profile. In contrast, in the *gacS* mutant CHA806, almost no β-galactosidase activity was measured (Fig. 2).

Impact of the *aprA* gene product on nematode populations. *Meloidogyne* spp., the root-knot nematodes, are sedentary endoparasites of a wide range of plants, including many of agronomical importance. *Meloidogyne incognita* belongs to a group of nematodes that cause important crop losses in developing countries (12, 19). Culture supernatants of wild-type strain CHA0 grown in 1/20-strength King's B medium (0.1% [wt/vol] Oxoid proteose peptone, 0.05% [wt/vol] glycerol, 0.3 mM

TABLE 3. In vitro effects of the addition of 4 mM EDTA to *P. fluorescens* culture filtrates on the mortality of *M. incognita* juveniles

Strain[a]	Juvenile mortality[b]	
	−EDTA	+EDTA
Control	16	19
CHA0	57*	33
CHA89	22	21
CHA805	19	24

[a] Culture filtrates of strains CHA0 (wild type), CHA89 (*gacA*), and CHA805 (*aprA'-'lacZ*) were grown in 1/20 King's B medium for 48 h. The control was made with sterile medium.
[b] Mortality is expressed as the percentage of dead juveniles after 24 h of exposure. Data for each strain and treatment were obtained from three different experiments with five repetitions. The mean of the experiment marked with an asterisk is significantly different ($P \leq 0.05$) from all the other values according to Fisher's test (least significant difference = 20%).

TABLE 4. Effects of carbofuran and *P. fluorescens* strains on gall formation caused by *M. incognita* and on soil and root populations in tomato and soybean grown under glasshouse conditions[a]

Strain or statistic	Galls/g root[b]		*M. incognita* organisms/g root[c]		*M. incognita* organisms/100 cm^3 soil	
	Tomato	Soybean	Tomato	Soybean	Tomato	Soybean
Control	101a	81a	136a	121a	1,524a	1,330a
Carbofuran	34c	29c	62c	52c	625c	380c
CHA0	62b	46bc	82bc	80b	1,238b	1,070b
CHA89	86ab	69ab	116a	107a	1,461a	1,196ab
CHA805	76ab	55b	107ab	97ab	1,456a	1,218ab
LSD[d]	26	23	30	25	164	153

[a] Sandy loam soil (sand:silt:clay, 70:19:11; pH 8.1; moisture holding capacity, 39%) from Karachi was placed in plastic pots (8-cm diameter). After removing the upper 2-cm layer of soil, 2.2×10^8 to 3.1×10^8 CFU ml^{-1} of bacteria suspended in 25 ml sterile 100 mM $MgSO_4$ or 120 mg kg^{-1} carbofuran (a granular nematocide purchased from Pak Agrochemicals, Karachi, Pakistan) was drenched. Soil drenched with 25 ml of sterile 100 mM $MgSO_4$ was used as a control. After treatment, three tomato seedlings (cv. SUN 6002 PVP) were planted in each pot. After 1 week, 2×10^3 *M. incognita* juveniles were added to each pot. The suspension was adjusted to 500 juveniles ml^{-1} by adding tap water, and a total of 4 ml was pipetted into four holes made around the seedlings in the soil. In another series of pots, after bacterial and chemical treatments, eight soybean (*Glycine max* L. cv. NARC I) seeds were sown in each pot, and after germination four seedlings per pot were retained. One week after emergence, the seedlings were infested with *M. incognita* juveniles as described above.

[b] Gall formation is expressed as the number of galls per gram of root after 45 days.

[c] *M. incognita* populations are expressed as the number of individuals per gram of root or per 100 cm^3 of soil (16, 22) after 45 days. Data for each strain and plant system were obtained from four different experiments with four repetitions. Means of the experiments in each column followed by different letters are significantly different ($P \leq 0.05$) according to Fisher's test.

[d] LSD, least significant difference.

$MgSO_4$, 0.3 mM K_2HPO_4) inhibited egg hatching and caused mortality of the juveniles of *M. incognita* in vitro, in comparison with the uninoculated controls ($P \leq 0.05$) (Table 2). The protease-negative mutants CHA805 (*aprA*) and CHA89 (*gacA*) failed to inhibit egg hatching and to kill *M. incognita* juveniles (Table 2). The addition of the protease inhibitor EDTA (4 mM) to a culture supernatant of strain CHA0 grown in King's B medium markedly reduced ($P \leq 0.05$) the juvenile killing activity of strain CHA0 but had little effect on the supernatants of the mutants CHA805 and CHA89 (Table 3). These data support the involvement of AprA protease in the inhibition of egg hatching and in killing of juveniles. However, AprA protease may not be the only antinematode factor of strain CHA0, in that antibiotic compounds produced under GacA control may also have a role in nematode control (23; I. A. Siddiqui and S. S. Shaukat, unpublished data).

In comparison to nonbacterized controls, *P. fluorescens* CHA0 applied to unsterilized sandy loam soil suppressed ($P \leq 0.05$) root-knot development and nematode final population densities on both tomato and soybean under greenhouse conditions (Table 4). Carbofuran (Furadan) treatment, however, was more effective in reducing nematode population densities in soil and roots and subsequent root-knot development in both crops (Table 4). Strains CHA805 and CHA89 had no significant impact on nematode population densities in soil and root-knot disease in either crop (Table 4). Application of strain CHA805 resulted in a reduction ($P \leq 0.05$), but not a complete loss, of nematode final population densities in soybean roots (Table 4). In these experiments, bacterial colonization of the tomato and soybean rhizospheres was not significantly different between the three strains tested (data not shown).

In conclusion, these findings are consistent with the notion that AprA protease of strain CHA0 contributes, directly or indirectly, to biocontrol of *M. incognita*. This study also extends previous observations that *P. fluorescens* CHA0 has biological control activity against root-knot nematodes (23–25).

Nucleotide sequence accession number. The 6.7-kb SacI-BamHI fragment of strain CHA0 was sequenced in this study and was deposited in GenBank under accession no. AY644718.

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