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Brief report

Collimonas fungivorans, an unpredicted *in vitro* but efficient *in vivo* biocontrol agent for the suppression of tomato foot and root rot

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Summary

Although bacteria from the genus *Collimonas* have demonstrated *in vitro* antifungal activity against many different fungi, they appeared inactive against the plant-pathogenic fungus *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*Forl*), the causal agent of tomato foot and root rot (TFRR). Visualization studies using fluorescently labelled organisms showed that bacterial cells attached extensively to the fungal hyphae under nutrient-poor conditions but not in glucose-rich Armstrong medium. *Collimonas fungivorans* was shown to be as efficient in colonizing tomato root tips as the excellent colonizer *Pseudomonas fluorescens* strain WCS365. Furthermore, it appeared to colonize the same sites on the root as did the phytopathogenic fungus. Under greenhouse conditions in potting soil, *C. fungivorans* performed as well in biocontrol of TFRR as the well-established biocontrol strains *P. fluorescens* WCS365 and *Pseudomonas chlororaphis* PCL1391. Moreover, under biocontrol conditions, *C. fungivorans* did not attach to *Forl* hyphae colonizing plant roots. Based on these observations, we hypothesize that *C. fungivorans* mainly controls TFRR through a mechanism of competition for nutrients and niches rather than through its reported mycophagous properties, for which attachment of the bacteria to the fungal hyphae is assumed to be important.

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Introduction

Soil bacteria from the genus *Collimonas* (De Boer *et al*., 2004) have demonstrable chitinolytic and antifungal activity. Originally retrieved from the top soil in between tussocks of *Ammophila arenaria* (marram grass), *Collimonas* isolates produced clear haloes on water-agar plates containing colloidal chitin (De Boer *et al*., 1998). In addition, they inhibited the exploratory growth from potato-dextrose agar plugs onto the surface of water-agar plates of several fungi, including *Chaetomium globosum*, *Fusarium culmorum*, *Idriella bolleyi*, *Mucor hiemalis*, *Phoma exigua* and an *Ulocladium* species (De Boer *et al*., 1998). Also included in this analysis was *Fusarium oxysporum*, but its growth was not affected by any of the *Collimonas* isolates tested (De Boer *et al*., 1998). Another property of *Collimonas* bacteria is their apparent ability to grow at the expense of living fungal hyphae (De Boer *et al*., 2001). Dubbed 'bacterial mycophagy' (Fritsche *et al*., 2006), this property manifested itself as an increase in the number of colony-forming units of *Collimonas* bacteria after inoculation into microcosms of sterilized purified sand that contained growing hyphae of *M. hiemalis* or *Chaetomium globosum* (De Boer *et al*., 2001). It is presently unknown what mechanisms underlie the mycophagous phenotype of *Collimonas* bacteria, but it has been suggested that attachment to fungal hyphae and chitinolytic activity are contributing factors. This is based on microscopic observations (De Boer *et al*., 2001) and the negative effect of the chitinase inhibitor allosamidin on the ability of *Collimonas* to grow on fungal hyphae in purified sand microcosms (De Boer *et al*., 2001).

Tomato foot and root rot (TFRR), a disease occurring worldwide, causes serious economical losses in the horticultural sector (Jarvis, 1988; Jones *et al*., 1991). Hyphae of the phytopathogenic fungus *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*Forl*) attach to tomato root hairs, colonize the root surface, and penetrate the internal root parts where a highly branched mycelium develops (Lagopodi *et al*., 2002). The infection

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process is accompanied by the development of lesions, rotting of the root and eventually leads to plant death. Chemical fungicides are not effective in suppressing TFRR (Benhamou *et al*., 1994). Moreover, their use has a negative connotation with media and policy makers. As an alternative to chemicals, beneficial microbes can be used for the biological control of soil-borne root diseases. These can employ various mechanisms of action such as production of antibiotic(s), volatiles and siderophores (Tomashow and Weller, 1995; Lugtenberg and Bloemberg, 2004; Haas and Defago, 2005), competition for nutrients and niches (Lemanceau and Alabouvette, 1990; Kamilova *et al*., 2005; Validov *et al*., 2006), induction of systemic resistance in the plant by triggering protection mechanisms (Pieterse *et al*., 1996; Van Loon *et al*., 1998; Kamilova *et al*., 2005), and predation and parasitism (Harman *et al*., 2004; Bolwerk, 2005). The best-known organism which uses predation and parasitism as a major mechanism is the fungus *Trichoderma* that produces extracellular cell wall-degrading enzymes (Woo *et al*., 1999; Brunner *et al*., 2003) such as glucan 1,3-β-glucosidases, N-acetyl-β-glucosaminadases (Lorito *et al*., 1994); chitobiosidases and endochitinases (Harman *et al*., 1993). Also several strains of *Serratia plymuthica*, *S. marcescens* and *S. liquefaciens* (Chet *et al*., 1990; Stanley *et al*., 1994; Kalbe *et al*., 1996; Kurze *et al*., 2001; Ovadis *et al*., 2004; Roberts *et al*., 2005) with strong chitinolytic activity appear to be good biocontrol agents. Given the ability of *Collimonas* to produce chitinolytic enzymes, its antifungal activity and mycophagous phenotype, we tested the potential of *Collimonas* as an adequate biocontrol agent of TFRR. For this we used *Collimonas fungivorans* Ter331, a strain that has been described in detail taxonomically (De Boer *et al*., 2004).

Results and discussion

In vitro *antagonistic tests*

In routine *in vitro* antagonistic tests (Kamilova *et al*., 2005) on solid potato dextrose (Difco Laboratories), Czapek-Dox (Difco Laboratories), Waksman (Berg, 2000), and King's B (King *et al*., 1954) media or on water agar (De Boer *et al*., 1998), *C. fungivorans* Ter331 did not inhibit the growth of *Forl*. This is in line with previous results (De Boer *et al*., 1998). In *in vitro* tests for the production of proteases (Brown and Foster, 1970), lipase (Howe and Ward, 1976), cellulase (Hankin and Anagnostakis, 1977) and β-glucanase (Walsh *et al*., 1995), *C. fungivorans* revealed activities only in the first two tests. The bacterium did not produce the broad-spectrum antimicrobial compound hydrogen cyanide in a test described by Castric (1975).

Visualization of the in vitro *interaction between* C. fungivorans *and* Forl

To visualize the interaction between *Collimonas* and *Forl* at the microscopic level, *C. fungivorans* strain Ter331 was transformed with plasmid pPROBE-NT-trp (Hallmann *et al*., 2001) to constitutively express green fluorescent protein and subsequently confronted with *cfp*-tagged *Forl* (Bolwerk *et al*., 2005), on glass slides covered with a thin layer of solidified medium. On Armstrong medium (Singleton *et al*., 1992) which contains 2% (w/v) glucose, fungal mycelium and cells of *Collimonas* showed no obvious interaction (Fig. 1A). When phosphate-buffered saline (PBS) without carbon source was used, we observed abundant colonization of developed fungal hyphae by bacterial cells (Fig. 1B). On agar containing tomato root exudate as the sole carbon source, prepared according to Kamilova and colleagues (2005), colonization of hyphae by bacteria was also observed, but to a lesser extent than in PBS (Fig. 1C). Tomato root exudate contains various organic acids and sugars (Lugtenberg *et al*., 2001; Kamilova *et al*., 2006), but the total amount of carbon in exudates is approximately 300 times lower than in Armstrong medium. Based on these observations, we hypothesize that low nutrient availability stimulates *Collimonas* to colonization fungal hyphae.

C. fungivorans *is a good colonizer of tomato roots*

Efficient competitive root colonization is important for the success of bacteria in their action against soil-borne phytopathogenic fungi (Chin-A-Woeng *et al*., 2000; Kamilova *et al*., 2005). Therefore we tested the competitive root colonization ability of *C. fungivorans*. As a criterion for good competitive root colonization, Simons and colleagues (1996) have developed an assay in which two strains are coated in a 1:1 ratio on seeds. After 1 week of seedling growth, the ratio of the two strains retrieved from various parts of the root is determined and used as a relative measure for competitive root colonization ability. In a competitive tomato root colonization assay of *C. fungivorans* Ter331 with *Pseudomonas fluorescens* strain PCL1285, a kanamycin-resistant derivative of the excellent tomato root colonizer *P. fluorescens* strain WCS365 (Lugtenberg *et al*., 2001), the cell numbers of Ter331 and PCL1285 on all parts of the root were statistically indistinguishable, i.e. their ratio was close to 1 (Table 1). These data demonstrate that *C. fungivorans* has excellent competitive tomato root-colonizing properties which in theory would allow it to deliver antifungal compounds such as cell wall-degrading enzymes or antibiotics and/or compete with pathogens for nutrients and niches on the plant root.

The ability of *C. fungivorans* to colonize the root as efficiently as the highly rhizosphere-competent strain

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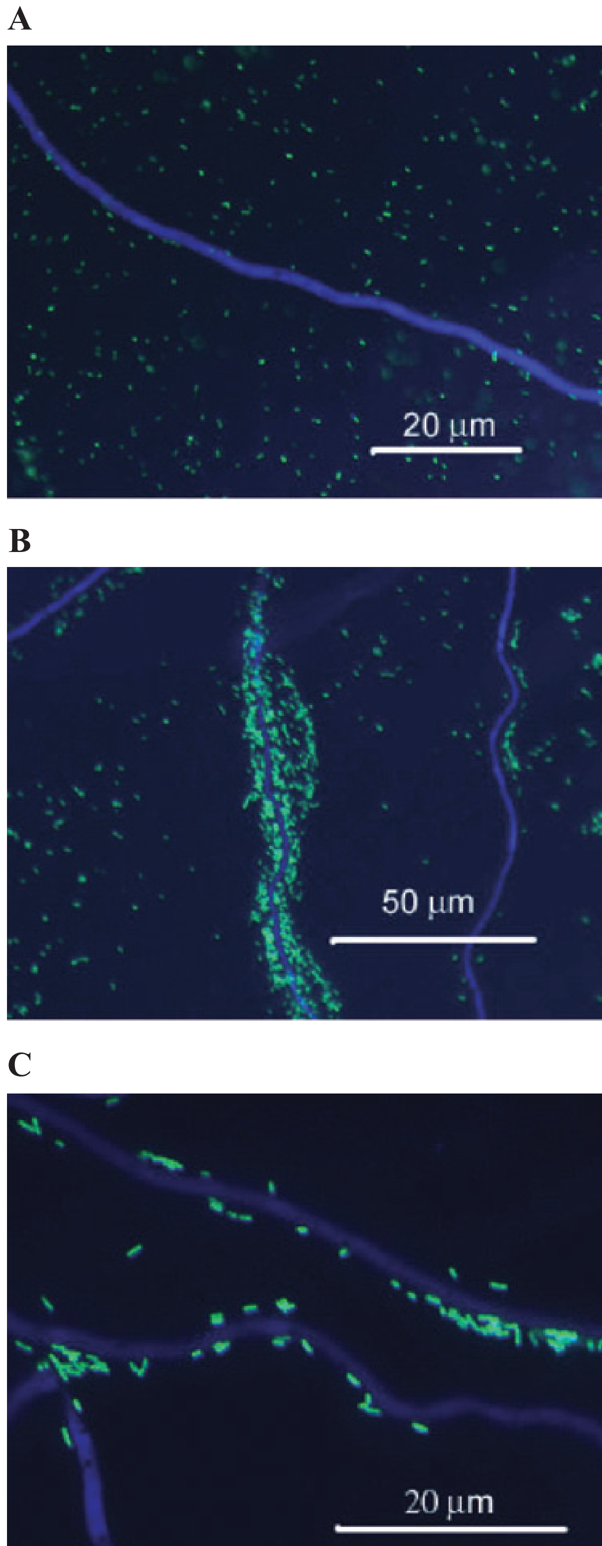

Fig. 1. Visualization of the *in vitro* interaction between *gfp*-tagged *C. fungivorans* and *cfp*-tagged *ForI*. Glass slides (25 × 15 × 1.5 mm) were prepared by spreading 150 μl (A) 1.8% Armstrong agar (glucose 20 g; KCl 1.6 g; KH_2PO_4 1.1 g; $Ca(NO_3)_2$ 5.9 g; $MgSO_4 \times 7H_2O$ 400 mg; microelements 200 μl from each stock solutions). Microelements stock solutions: $FeCl_3$ 167 mg/100 ml; $MnSO_4$ 107 mg/100 ml; $ZnSO_4$ 178 mg/100 ml); (B) PBS agar, or (C) agar containing tomato root exudate collected according to Kamilova and colleagues (2005). Two-millimetre-diameter plugs of *ForI* grown on Armstrong plates were placed on the agar layer of the corresponding glass slides. Bacteria were overnight grown in 1/20 tryptic soy broth and washed twice in 0.9% NaCl. Bacteria were spotted as a 10 μl drop (10^6 cells) at the distance of 1 cm from the plug of *Fusarium*. Slides were incubated prior to microscopy for 3 days at 28°C in Petri dishes lined with wet filter paper and sealed with parafilm to prevent drying. Each treatment consisted of five slides and the experiment was performed twice.

P. fluorescens WCS365 is surprising because *C. fungivorans* grows slower than WCS365 *in vitro* (data not shown). Apparently, the tomato rhizosphere provides the bacterium with conditions which allow it to compete with WCS365.

Microscopic visualization of colonization of tomato root by C. fungivorans *under gnotobiotic conditions*

Confocal laser scanning microscopy of tomato roots grown from seeds coated with the *gfp*-tagged *C. fungivorans* Ter331 revealed a pattern of root colonization in which the numbers of bacterial cells gradually decreased from the foot to the root tip (results not shown). A similar pattern of tomato root colonization was observed earlier for *P. fluorescens* strain WCS365 (Dekkers *et al.*, 2000) and for *Pseudomonas chlororaphis* strain PCL1391 (Bolwerk *et al.*, 2003). Microcolonies of *Collimonas* were observed mostly in intracellular junctions (Fig. 2A and B)

Table 1. Competitive tomato root tip colonization of *C. fungivorans* and *P. fluorescens* PCL1285, kanamycin-resistant derivative of *P. fluorescens* WCS365.

Competitive root colonization[a]		
	Competing strains	
Analysed root part[b]	PCL1285[c]	*C. fungivorans*[c]
Foot	5.97 ± 0.15 (a)	5.84 ± 0.09 (a)
Middle part	5.67 ± 0.70 (a)	5.07 ± 0.43 (a)
Root tip	4.90 ± 0.85 (a)	4.69 ± 0.65 (a)

a. In every experiment, 10 plants were inoculated. When values from the same experiment are followed by a different letter, they are significantly different at the $P = 0.05$ level, according to the Wilcoxon Mann–Whitney test.
b. Bacterial strains were inoculated according to Simons and colleagues (1996) on tomato seedlings cv Carmello in a 1:1 ratio. Plant roots were isolated 7 days after inoculation. One-centimetre sections from foot, middle part and root tip of each root were analysed for bacterial population sizes.
c. Data are expressed as $\log_{10}$[(CFU + 1)/cm of root].

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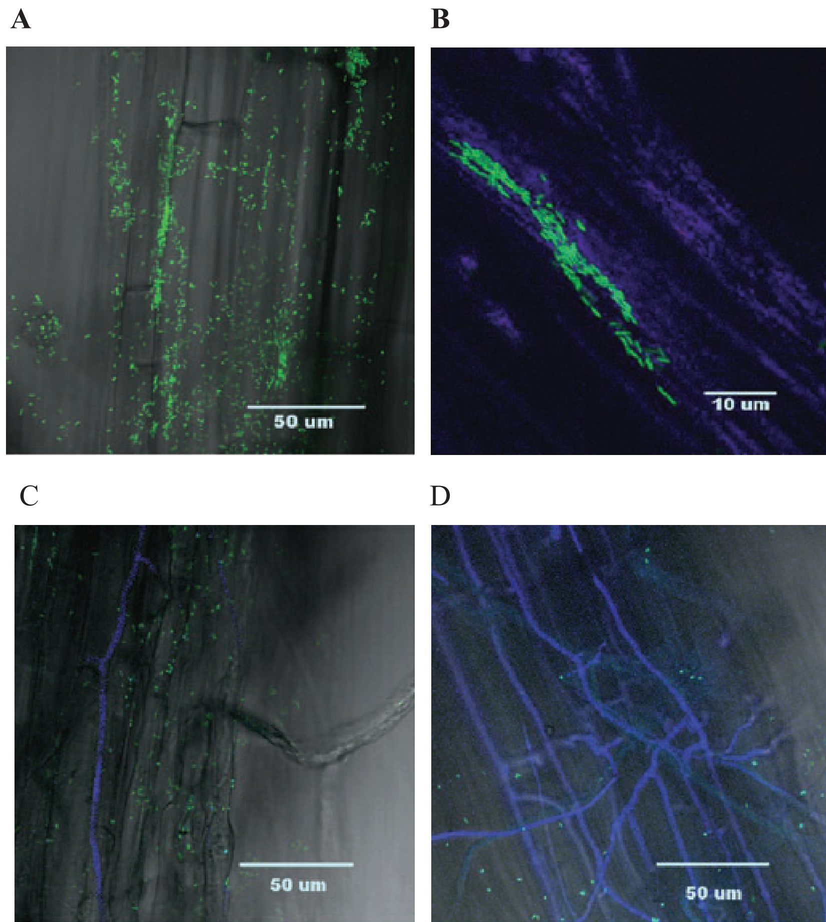

Fig. 2. Confocal laser scanning microscopical analysis of tomato root colonization by *C. fungivorans* Ter331 and phytopathogenic fungus *F. oxysporum* f.sp. *radicis-lycopersici* (*Forl*). Two-day-old tomato seedlings were inoculated with cells of *C. fungivorans* expressing the *gfp* gene, which here appear as green cells. Plants were grown in gnotobiotic sand system containing spores of *Forl* harbouring a constitutively expressed *cfp* gene (5×10^3 spore per kg sand). Developed hyphae here appear as blue. Tomato plants were grown for 7 days in a plant-growth chamber at 24°C, 70% humidity, and cycles of 16 h light/8 h dark. A total of 10 plants per treatment were examined and the experiment was repeated twice. At least 10 different fields of view were observed per root. Low-magnification represents the view of colonization by *C. fungivorans* of a plant root grown in untreated sand (A); high-magnification represents a microcolony of *C. fungivorans* (B). Colonization by *C. fungivorans* and *Forl* of a root that showed no macroscopically visible lesions and that was scored as healthy (C). Colonization by *C. fungivorans* and *Forl* of a root that showed lesions and was scored as being sick (D).

as was observed earlier (Bolwerk *et al*., 2003) for the biocontrol *Pseudomonas* strains mentioned previously.

Biocontrol of TFRR

In four independent experiments (for details, see legend of Table 2), coating of tomato seeds (Chin-A-Woeng *et al*., 1998) with cells of *C. fungivorans* led to significant biocontrol of TFRR (Table 2) in potting soil under greenhouse conditions. This beneficial effect of *C. fungivorans* is comparable with that of the well-established biocontrol strains *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 (Table 2). We have tested the ability of *C. fungivorans* to induce systemic resistance in tomato plants against *Forl* using a split root system as described by Kamilova and colleagues (2005). Being spatially separated from the disease-causing agent, *C. fungivorans* did not control TFRR whereas the positive control *P. fluorescens* WCS365 (Kamilova *et al*., 2005) did (data not shown), which show that *C. fungivorans* and *P. fluorescens* WCS365 differs in their mechanism of biocontrol of TFRR.

Under gnotobiotic biocontrol conditions in a quartz sand/plant nutrient solution (Hoffland *et al*., 1989) system, the density of fungal hyphae in each of 10 fields of view of the root surface was always lower when bacteria were present than when bacteria were not present (Fig. 2C). Roots that showed clear disease symptoms were colonized by bacteria to a much lesser extent than roots of healthy looking plants (Fig. 2D). Surprisingly, we did under no circumstances observe hyphal colonization by *Collimonas* cells on plant root surfaces in contrast to observations on the glass slides (Fig. 1B and C). We cannot exclude the possibility that on the root the bacterium attacks and lyses the hyphae much faster than *in vitro.* If that were the case, one would have expected intermediate stages of this process such as hyphae with many attached bacteria and morphologically altered hyphae. However, we did not observe these.

We conclude from our experiments that *C. fungivorans* is a biocontrol strain acting efficiently against *Forl* under greenhouse conditions. Our data on tomato split roots allow us to rule out induction of systemic resistance as a possible mechanism of TFRR biocontrol by *C. fungivorans*. Strong competitiveness of *Collimonas* with an excellent root colonizer *P. fluorescens* WCS365 and ability to colonize the same sites on the tomato roots that

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Table 2. Biocontrol of TFRR caused by *F. oxysporum* f.sp. *radicis-lycopersici*.[a]

Experiment number	Treatment			
	Negative control	*C. fungivorans*	WCS365	PCL1391
1	57 ± 15 (a)	43 ± 15 (b)	27 ± 15 (bc)	nd
2	51 ± 13 (a)	22 ± 9 (b)	21 ± 9 (b)	25 ± 9 (b)
3	60 ± 16 (a)	42 ± 15 (b)	nd	29 ± 8 (b)
4	56 ± 12 (a)	33 ± 6 (b)	30 ± 11 (b)	33 ± 10(b)

a. Overnight cultures of *C. fungivorans*, *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 grown in King's B medium were washed in PBS and adjusted to an optical density at 620 nm (OD_{620}) of 0.7. For coating the bacterial suspensions of OD_{620} of 0.7 were mixed with an equal volume of 2% (wt/vol) methylcellulose. Control seeds were coated with PBS mixed with equal volume of 2% methylcellulose. Tomato seeds were inoculated by immersion in the resulting suspensions for 10 min and air-dried. Biocontrol by the tested bacteria of TFRR was performed as described by Chin-A-Woeng and colleagues (1998). Coated tomato seeds were sown in *F. oxysporum* f.sp. *radicis-lycopersici* infested soil (2×10^6 spores per kg soil) in multicell plastic trays. In experiments N1 and N3, the negative controls comprised 24 replications, compared with 16 replications for treatments with *C. fungivorans*. In experiments N2 and N4, the negative controls comprised 16 replications and the treatment with *C. fungivorans* eight replications. In all experiments, treatments with WCS365 and PCL1391 comprised eight replications. Each replication for each treatment contained 12 plants. After incubation for 21 days in a climate-controlled growth chamber at 20°C and 70% relative humidity, the number of diseased roots was assessed. Data present the percentage of diseased plants ± standard deviation. Values indicated with different letters are statistically significantly different. Data were analysed for significance using analysis of variance followed by Fischer's least significant difference test ($a = 0.05$), using SPSS software (SPSS, Chicago).
nd, not done.

otherwise are occupied by *Fusarium* suggest competition for nutrients and niches as a likely mechanism of biocontrol. *Collimonas* shows an *in vitro* chitinolytic activity similar to that of biocontrol strain *S. plymuthica* (Berg, 1996). The latter also showed *in vitro* antagonism against causal pathogenic fungi *Verticillium dahliae* and *V. longisporum* and was efficient in biocontrol of *Verticillium* wilt of oilseed rape (Berg, 2000; Berg *et al*., 2000). Hence, *C. fungivorans* makes an interesting case, where an *in vitro* observation (i.e. no antifungal activity towards *Forl in vitro*, despite its *in vitro* chitinolytic activity) seems to be a false predictor of its actually efficient biocontrol activity *in vivo*.

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