

Brief note

Prevalence of type III secretion system in effective biocontrol pseudomonads

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Abstract

Functional type III secretion system (T3SS) genes are needed for effective biocontrol of *Pythium* damping-off of cucumber by *Pseudomonas fluorescens* KD, but whether biocontrol *Pseudomonas* strains with T3SS genes display overall a higher plant-protecting activity is unknown. The assessment of 198 biocontrol fluorescent pseudomonads originating from 60 soils worldwide indicated that 32% harbour the ATPase-encoding T3SS gene *hrcN*, which was most often found in tomato isolates. The *hrcN*⁺ biocontrol strains (and especially those also producing 2,4-diacetylphloroglucinol and displaying 1-aminocyclopropane-1-carboxylate deaminase activity) displayed higher plant-protecting ability in comparison with *hrcN*⁻ biocontrol strains, both in the *Pythium*/cucumber and *Fusarium*/cucumber pathosystems.

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1. Introduction

Many fluorescent *Pseudomonas* strains can control soil-borne crop diseases, often using antimicrobials e.g. phenazines, hydrogen cyanide, pyrrolnitrin and/or 2,4-diacetylphloroglucinol (Phl) [1–3]. Phl is of particular interest because (i) it inhibits a variety of soil-borne

phytopathogens [1,4], (ii) it can trigger induced systemic resistance [3,5], (iii) it induces transcription of phytostimulation-related genes in other plant-beneficial rhizobacteria [6], and (iv) Phl⁺ biocontrol *Pseudomonas* strains were shown to protect plants better than Phl⁻ counterparts in two pathosystems [7].

Other properties may also be involved in disease suppression by fluorescent pseudomonads, including the production of extracellular lytic enzymes, surfactants, siderophores, and (so far unknown) effectors secreted by the type III secretion system (T3SS) [1,8]. The implication of T3SS in biological control was shown in plant experiments, where inactivation of T3SS gene *hrcV* strongly diminished the ability of *Pseudomonas* sp. KD to protect cucumber from *Pythium* damping-off [8], but results with the take-all/wheat pathosystem were not

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as clear-cut following T3SS inactivation in *Pseudomonas* sp. Q81r1-96 [9]. In addition to *Pseudomonas* strains KD and Q81r1-96, T3SS genes have also been evidenced in other saprophytic pseudomonads [10–12] including Phl⁺ strains [9,13,14].

The significance of T3SS genes for biological control by pseudomonads is difficult to determine, because inactivation of T3SS genes has been performed only in two strains, which retained other biocontrol activities [8,9]. Indeed, *Pseudomonas* strains KD and Q81r1-96 can also produce Phl, hydrogen cyanide and siderophore(s). Furthermore, elimination of a particular biocontrol trait may alter the expression of the others in *Pseudomonas* [15].

The objective was to assess at population level whether or not *hrcN*⁺ biocontrol pseudomonads display higher plant-protecting activity than *hrcN*[−] counterparts. This was assessed using 198 biocontrol fluorescent pseudomonads derived from a collection of 230 biocontrol strains selected by screening several thousand *Pseudomonas* isolates [7]. We also compared the occurrence of T3SS gene with that of three other types of plant-beneficial properties, i.e. Phl synthesis [7], 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity [16] and surfactant production [17].

2. Materials and methods

2.1. Isolate origin and biocontrol activity

The 198 biocontrol pseudomonads (Table S1) originated from bulk soil (2 isolates) or macerated surface-disinfected roots of cucumber (98 isolates), tomato (40 isolates), wheat (34 isolates), tobacco (16 isolates), cotton (4 isolates), or bean (4 isolates) grown from surface-disinfected seeds planted in soils obtained from 60 geographic locations in 18 countries

[7]. Their biocontrol ability was documented in the *Pythium ultimum*/cucumber pathosystem in steam-pasteurized clay loam soil, and for most of them (i.e. 90%) also in the *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL)/tomato pathosystem in rockwool substrate [7].

2.2. *hrcN* screening and trait analyses

The 198 biocontrol pseudomonads were screened for the presence of the *hrcN* gene by PCR using primers *hrcN*-5rR/*hrcN*-4r, as described [13]. The PCR products obtained were purified and both strands sequenced [13]. *hrcN* identity was determined using BlastN and the nr Nucleotide Sequence Database. A phylogenetic analysis was conducted with the 64 partial *hrcN* sequences obtained (250 bp) and others already available. The flagellar ATPase gene *fliI* of *Pseudomonas putida* KT2440 was used as the outgroup. Sequences were aligned using MUSCLE [18], most informative positions were selected using Gblocks [19] and phylogenetic distances were calculated using the GTR model [20]. The maximum likelihood tree was inferred using PhyML [21] and nodal robustness was assessed using 500 bootstrap replicates. All steps were performed using Seaview software [22].

The ability to produce Phl and/or HCN had been determined by Rezzonico et al. [7] and the resulting data were used in this work. Phl had been found (by HPLC analysis, as described [4]) in 71 of the 198 biocontrol fluorescent pseudomonads (i.e. 36%) and HCN (by reaction with copper(II)-ethyl acetoacetate, as described by Castric & Castric [23]) in 144 of the 198 biocontrol fluorescent pseudomonads (i.e. 73%). Here, the capacity to deaminate ACC was scored based on the ability to use ACC as sole nitrogen source on DF salts minimal agar containing 3 mM ACC, as described by Wang et al. [16]. Surfactant production was assessed using the drop-collapse method [24].

Table 1

Prevalence of *hrcN*⁺ and *hrcN*⁺ Phl⁺ AcdS⁺ traits among the 198 biocontrol pseudomonads based on isolate origin and the capability for HCN production, Phl production, ACC deamination and surfactant production.

	Number of isolates	<i>hrcN</i>		<i>hrcN</i> ⁺ Phl ⁺ AcdS	
		<i>hrcN</i> ⁺ isolates ^a	<i>hrcN</i> [−] isolates	<i>hrcN</i> ⁺ Phl ⁺ AcdS ⁺ isolates ^b	Other isolates ^d
Origin	198	64	134	43	155
Cucumber	98	30	68	15	83
Tomato	40	21*	19	19#	21
Wheat	34	7	27	4	30
Tobacco	16	3	13	3	13
Other plants or soil	10	3	7	2	8
Biocontrol traits					
HCN production	145	58*	87	43#	102
Phl production	71	50*	21	43#	28
ACC deamination	85	51*	34	43#	42
Surfactant production ^c	26	0	26	0	26

^a In each row, * indicates a significantly higher proportion of *hrcN*⁺ isolates within the different classes for origin (cucumber, tomato, wheat, tobacco or other) or biocontrol traits (HCN production, Phl production, ACC deamination or surfactant production) than expected from the overall proportion (64 of 198 isolates) and a random association (χ^2 tests, $P < 0.05$).

^b In each row, # indicates a significantly higher proportion of *hrcN*⁺ Phl⁺ AcdS⁺ isolates within the different classes for origin or biocontrol traits than expected from the overall proportion (43 of 198 isolates) and a random association (χ^2 tests, $P < 0.05$).

^c The association between surfactant production and *hrcN* (or *hrcN* Phl AcdS) could not be tested since n was 0.

^d These other pseudomonads displayed two, one or none of the three traits.

2.3. Statistics

Two statistical approaches were followed. First, χ^2 tests of independence were done to test the association between the presence of *hrcN* (or combined traits) and other isolate features i.e. origin, presence of other biocontrol trait(s) and biocontrol performance. Second, pairwise comparisons of mean biocontrol performance levels were done using one-tailed Student *t* tests. All statistics were done at $P < 0.05$ using Systat version 10.0 (Systat Inc., Evanston, IL).

3. Results and discussion

3.1. Prevalence of TTSS gene *hrcN* and *hrcN* phylogeny

The T3SS gene *hrcN* was found in 64 of the 198 biocontrol pseudomonads (i.e. 32.3%), which is lower than in other biocontrol pseudomonads previously studied (i.e. 58.6% [13]). It was present in 19–31% of the isolates from wheat, tobacco and cucumber, but at a higher level (53%; χ^2 tests) in the

tomato isolates (Table 1). The prevalence of *hrcN* fluctuated according to the geographic origin of the isolates (Table S1) and reached as much as 45% in Swiss isolates, which were obtained from tomato or cucumber to a large extent. Phylogenetic analysis of partial *hrcN* sequences showed that the vast majority (i.e. 53) of the 64 *hrcN*⁺ biocontrol pseudomonads used in this work belonged to a large, rather homogeneous cluster (termed group 3 in Rezzonico et al. [13]) that contained also the biocontrol pseudomonads Q8r1-96 and TM1A3, as well as *Pseudomonas brassicacearum* NFM421 (Fig. S1). Unlike in Rezzonico et al. [13], group-2 sequences (such as in strains Q2-87 and PITR2) were not found with these strains. The other *hrcN*⁺ biocontrol pseudomonads from this work were distributed in the other clusters or were found outside Rezzonico's groups.

3.2. Prevalence of other plant-beneficial traits

Previous work with these pseudomonads showed that Phl production ability was an important attribute for superior plant

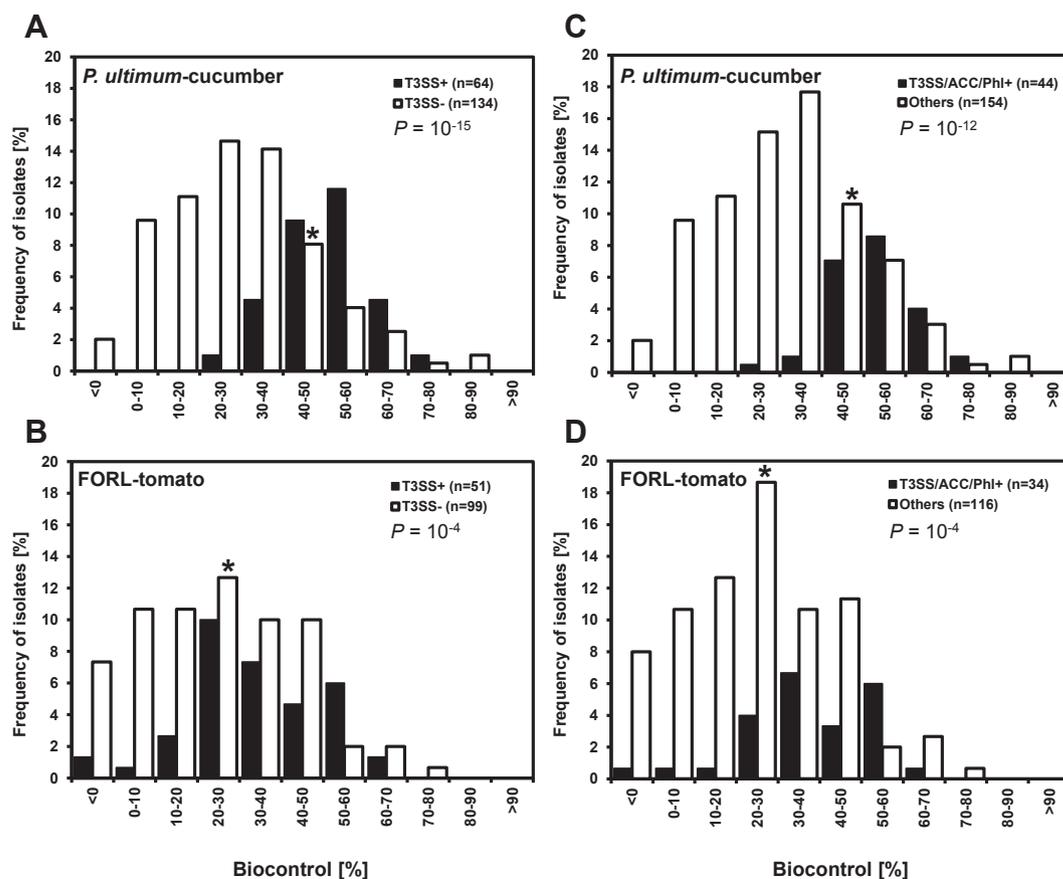


Fig. 1. Distribution of *Pseudomonas* isolates with respect to their biocontrol activity in the *P. ultimum*/cucumber (A, C) and FORL/tomato (B, D) pathosystems. Pseudomonads that are *hrcN*⁺ (black bars in A and B) or *hrcN*⁺ Phl⁺ AcdS⁺ (black bars in C and D) were compared with isolates that are respectively *hrcN*⁻ (white bars in A and B) or do not share all three properties (white bars in C and D) in each particular pathosystem. The biocontrol activity was computed based on plant fresh weight (*P. ultimum*-cucumber) or disease index (FORL-tomato) and the asterisk indicates the position of *hrcN*⁻ *P. protegens* strain CHA0 [4] used as a reference biocontrol agent over the six years of the study (41.2% in the *P. ultimum*/cucumber pathosystem and 29.6% in the FORL/tomato pathosystem). In the *P. ultimum*/cucumber pathosystem, biocontrol activity (%) was calculated as $(1 - ((WC - WI)/(WC - WP))) \times 100$ using plant fresh weight obtained in presence of the isolate tested (WI), in the control with no microorganisms added (WC) and in the unprotected control with the pathogen alone (WP). In the FORL/tomato pathosystems, biocontrol activity (%) was calculated as $(1 - ((IC - I)/(IC - IP))) \times 100$ in which *I*-values represent disease indices. The *P* values at which the two distributions differed in χ^2 tests are shown within each graph.

protection [7]. Here, the ability to deaminate ACC and produce surfactant was found in 89 (i.e. 45%) and 28 strains (i.e. 14%), respectively (Table 1), confirming that ACC deaminase activity is widespread in biocontrol pseudomonads [16,25]. The prevalence of *hrcN*, which reached 32% overall, was significantly higher in the biocontrol pseudomonads capable of producing Phl (70%), deaminate ACC (60%) or produce HCN (40%), whereas *hrcN* was not found in strains producing surfactant (Table 1). Thus, a large proportion of the *hrcN*⁺ biocontrol pseudomonads could produce Phl (78%), deaminate ACC (i.e. AcdS⁺; 80%) or produce HCN (91%), and 43 of the 64 *hrcN*⁺ biocontrol pseudomonads (i.e. 58%) were also Phl⁺, AcdS⁺ and HCN⁺.

3.3. Relation between *hrcN* presence and biocontrol

In the two pathosystems studied (Fig. 1A,B), the presence of *hrcN* in the biocontrol pseudomonads was associated with superior plant protection level based on χ^2 tests. The higher efficacy of *hrcN*⁺ pseudomonads was also indicated by one-tailed *t* tests. This was shown both in the *P. ultimum*/cucumber pathosystem (i.e. biocontrol level of $50.8 \pm \text{SE } 1.4\%$ versus $28.4 \pm 1.6\%$; $P = 10^{-15}$), where the importance of T3SS genes in *Pseudomonas* sp. KD was evidenced [8], and the FORL/tomato pathosystem ($34.2 \pm 2.3\%$ versus $23.3 \pm 1.9\%$; $P = 10^{-4}$). When considering the most effective pseudomonads, 82.8% of *hrcN*⁺ isolates exceeded a 40% biocontrol level in the *P. ultimum*/cucumber pathosystem (versus only 23.8% for *hrcN*⁻ isolates), whereas 56.8% of *hrcN*⁺ isolates exceeded a 30% biocontrol level in the FORL/tomato pathosystem (versus only 37.3% for *hrcN*⁻ isolates) (one-tailed *t* tests).

When considering not only *hrcN* but also the combination of different biocontrol traits, *hrcN*⁺ Phl⁺ AcdS⁺ pseudomonads (which were also HCN⁺) performed better overall (one-tailed *t* tests) than the other isolates in the *P. ultimum*-cucumber (i.e. biocontrol level of $53.5 \pm 1.6\%$ versus $30.5 \pm 1.5\%$, $P = 10^{-12}$) and the FORL-tomato systems ($38.2 \pm 2.8\%$ versus $23.7 \pm 1.7\%$, $P = 10^{-4}$). This was also indicated by χ^2 tests (Fig. 1C,D). When limiting the comparison to the best performers, 93.2% and 73.5% of the *hrcN*⁺ Phl⁺ AcdS⁺ isolates exceeded 40% and 30% biocontrol levels in the *P. ultimum*/cucumber and FORL/tomato pathosystems, respectively, which was higher (one-tailed *t* tests) than for the other isolates (28.6% and 35.3%, respectively).

3.4. Conclusion

This work showed that biocontrol pseudomonads with the T3SS gene *hrcN* protected better than *hrcN*⁻ biocontrol pseudomonads in the two pathosystems tested. The most efficient strains were often Phl⁺ AcdS⁺ in addition to *hrcN*⁺, pointing to the usefulness of combined plant-beneficial traits in these bacteria.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.resmic.2014.03.008>.

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