

REVIEW

An Overview of Recent Genomic Research on Biocontrol *Pseudomonad* Strains Isolated from the Field in Japan

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Abstract

Many root-colonizing *Pseudomonas* spp. exhibiting biocontrol activities produce a wide variety of secondary metabolites with antibiotic activity against plant pathogens in the rhizosphere. Among these metabolites, 2,4-diacetylphloroglucinol (DAPG) is one of the most typical antibiotics in model biocontrol strains of *Pseudomonas protegens*. We screened DAPG-producing strains among 2,800 fluorescent pseudomonads isolated from the field in Japan. In addition to *P. protegens*, several other strains close to, but different from, *P. protegens* have been identified and found to exhibit biocontrol activity. Recent advances in genomic research on these strains revealed that they build up strain-specific genomic repertoires for the biosynthesis of secondary metabolites and niche adaptation. In this mini-review, we introduced our recent genome-based characterization of *P. protegens* and related strains. Comparative genome analyses in combination with different bioassays have become a powerful tool for the identification of novel biocontrol factors of potentially beneficial strains.

Discipline: Plant protection

Additional key words: biocontrol, genome analysis, pseudomonads, rhizosphere, secondary metabolites

Introduction

Plant diseases caused by soil-borne pathogens, such as fungi, oomycetes, nematodes, and insects, have major negative impacts on crop growth and yield. Previous studies on rhizosphere microbial communities have provided critical information on the plant-beneficial microbes responsible for plant health from an ecological point of view (Vacheron et al. 2013). Many root-colonizing pseudomonads classified into the *Pseudomonas fluorescens* group are effective biocontrol strains that suppress plant diseases in the rhizosphere. The *P. protegens* strains CHA0 and Pf-5 (previously called *P. fluorescens* CHA0 and Pf-5, respectively) have been used as model strains in studies on the biosynthesis of secondary metabolites, such as 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (Plt), with antibiotic activities in the rhizosphere (Haas & Keel 2003). These exoproducts contribute to plant protection by these strains and other

root-colonizing *Pseudomonas* species with biocontrol activity.

P. protegens CHA0 was isolated from the roots of tobacco in Swiss soil, which was naturally suppressive to black root rot in tobacco caused by *Thielaviopsis basicola* (Stutz et al. 1986). The molecular mechanisms underlying the expression of biocontrol factors depending on the Gac/Rsm signal transduction pathway were elucidated by the group of Dieter Haas (Lapouge et al. 2008 and the references cited therein). The Gac/Rsm cascade is initiated by the GacS/GacA two-component system, and activated GacA then promotes the transcription of non-coding small RNAs (sRNAs) termed RsmX, RsmY, and RsmZ. These sRNAs have high affinity for the RNA-binding proteins RsmA and RsmE in strain CHA0. RsmA/E proteins repress the translation of genes involved in secondary metabolism during trophophase. When sRNAs are induced, they relieve the translational repression of target genes by sequestering the RsmA and RsmE proteins, thereby

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allowing the synthesis of secondary metabolites. Thus, mutants defective in the Gac/Rsm signal transduction pathway have a reduced ability to produce biocontrol factors and suppress plant diseases.

Strain Pf-5 was isolated from the rhizosphere soil surrounding cotton seedlings in Texas, USA (Howell & Stipanovic 1979). Since the whole-genome sequence of strain Pf-5 has been deciphered (Paulsen et al. 2005), a large amount of genomic information on biocontrol pseudomonads has been reported, which has advanced biocontrol research into a new era. The availability of the genome sequences of pseudomonads has facilitated research on the identification of genes involved in the biosynthesis of secondary metabolites. The group of Loper and Gross has focused on the discovery of novel biocontrol factors from orphan biosynthetic gene clusters (Gross & Loper 2009). Besides the typical gene clusters encoding the synthesis of biocontrol factors, such as *phl* (for DAPG), *plt* (for pyoluteorin), *prn* (for pyrrolnitrin), *hcn* (for hydrogen cyanide), *apr* (for exoprotease AprA), *pvd* (for pyoverdine), and *pch* (for enantio-pyochelin), genome data have led to the discovery of other important gene clusters, including the rhizoxin analog biosynthesis gene cluster (*rxz*) consisting of genes encoding polyketide synthase (Loper et al. 2008). Novel cyclic lipodecapeptide orfamide A (Gross et al. 2007) and the insect toxin FitD (Péchy-Tarr et al. 2008) have also been discovered through genomic-guided approaches using strains Pf-5 and CHA0.

Although *P. protegens* has been isolated from North America, Europe, and Africa (Keel et al. 1996, Ramette et al. 2011), limited information is currently available on the characterization of *P. protegens* in Asia. We recently attempted to screen biocontrol pseudomonad strains relative to strains Pf-5 and CHA0 from the field in Japan, with the expectation that their genome data will contribute to studies on the genomic diversity of the strains and the identification of factor(s) involved in the biocontrol efficacy of newly isolated strain(s).

In terms of the effective and ecological applications of biocontrol agents, screening for plant commensal pseudomonads has led to advances in plant protection research. In this review, we will introduce our recent genomic research on biocontrol pseudomonad strains relative to *P. protegens* isolated from the field in Japan.

Isolation of pseudomonads from rhizosphere soil and selection for biocontrol strains

We isolated approximately 2,800 fluorescent pseudomonads based on a previously described method (Someya et al. 2012, Figure 1). Among the secondary

metabolites described above, DAPG is the most typical biocontrol factor in *P. protegens* and is easy to detect. Therefore, we pre-screened DAPG-producing isolates by PCR using DAPG biosynthesis gene *phlD*-specific primers. On the basis of this analysis, 48 isolates were selected as candidates of DAPG-producing isolates, and their production activities were confirmed by thin-layer chromatography. Five out of the 48 strains were PCR-positive for the three antibiotic biosynthetic genes tested (*plt*, *prn*, and *hcn*). One of the five strains, named Cab57, which was isolated from the rhizosphere of shepherd's purse [*Capsella bursa-pastoris* (L.) Medik.] growing in a field in Hokkaido, was selected for further analyses. Cab57 was identified as *P. protegens* based on a 16S rRNA gene analysis (with 100% identity) and whole-genome analysis (Takeuchi et al. 2014).

Among the remaining strains, 39 were PCR-positive for the hydrogen cyanide biosynthesis gene, but negative for the pyrrolnitrin and pyoluteorin biosynthesis genes. On the basis of the analysis of 16S rRNA sequence variations, seven of these strains were found to have 99% similarities in their 16S rRNA gene with that of *P. protegens*, which was the highest homology among the sequenced strains. Among the seven strains, the 16S rRNA sequences of five strains, all of which are from the rhizosphere of rice [*Oryza sativa* L.] growing in a paddy field in Ibaraki, showed 100% identity. We selected one of these five strains, named Os17, for further analyses.

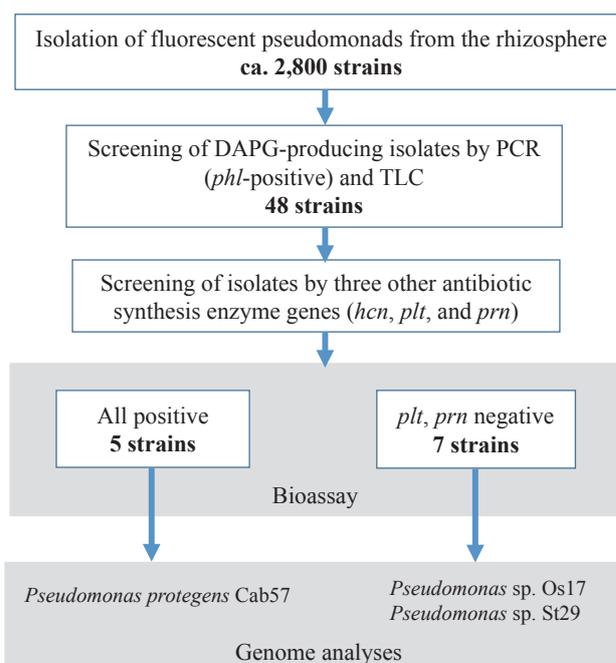


Fig. 1. Scheme for screening and characterization of strains with biocontrol efficacy and strains for genome analyses

We also selected one of the remaining two strains, named St29, which was isolated from the rhizosphere of potato [*Solanum tuberosum* L.] growing in a field in Ibaraki. Strains Os17 and St29 shared 99.94% identity in their 16S rRNA, suggesting that these two strains belong to the same species.

Biocontrol efficacy of strains Cab57, Os17, and St29

In order to investigate the biocontrol activities of strains Cab57, Os17, and St29 in a natural habitat, we adopted a cucumber-*Pythium ultimum* pathosystem (Haas & Keel 2003 and the references cited therein), which enabled us to evaluate plant protection efficacy by measuring root and shoot weights (Takeuchi et al. 2014, 2015). Strains Cab57 and Os17 were as effective biocontrol agents as strain CHA0, whereas strain St29 was less effective than the other strains (Table).

Genome structures of Cab57, Os17, and St29

We conducted whole-genome sequencing to obtain information on strains Cab57, Os17, and St29 using paired-end and whole-genome shotgun sequencing. All genomes were organized into a single circular chromosome with ca. 6.8-6.9 Mbp. The general genomic features of these strains as well as those of strains CHA0

and Pf-5 are listed in the table. Cab57 showed properties typical of *P. protegens*.

In order to define species based on whole-genome data, the JSpecies program has been commonly used as a recent standard (Richter & Rosselló-Móra 2009), therefore we applied this program for species identification of these three strains. The average nucleotide identity calculated with BLAST algorithm (ANIb) values, which provides a numerical and stable species boundary, confirmed the adscription of strain Cab57 to *P. protegens*, whereas Os17 and St29 were not identified as *P. protegens*. This program also confirmed the categorization of strains Os17 and St29 into the same species (Takeuchi et al. 2015).

A BLASTp search of strains Os17, St29, and Cab57 revealed that unique coding sequences (CDSs) were the most abundant in strain Cab57 (736), whereas they were 256 and 347 in strains Os17 and St29, respectively, reflecting the heterology of strain Cab57 among the three strains (Takeuchi et al. 2014, 2015). Strains Os17 and St29 shared 519 CDSs, except for core genes (5,321), some of which may reflect the species-specific repertoire, as described below. We employed the antiSMASH program to predict secondary metabolite biosynthetic gene clusters (Blin et al. 2013).

Regarding biocontrol factors, two antibiotic biosynthesis gene clusters (*phl* and *hcn*) were conserved in the genomic sequences of strains Os17 and St29, whereas two other gene clusters (*plt* and *prn*) were absent, which

Table 1. General genomic features and comparison of plant protection efficacies of *Pseudomonas protegens* strains Pf-5, CHA0, and Cab57 and *Pseudomonas* sp. strains Os17 and St29

	Pf-5*	CHA0**	Cab57***	Os17****	St29****
Genome size (bp)	7,074,893	6,867,980	6,827,892	6,885,464	6,833,117
Coding sequence number	6,108	6,115	6,186	6,195	6,217
G+C content (%)	63.3	63.4	63.3	63.5	63.3
Gene clusters encoding typical biocontrol factors	<i>phl, hcn, plt, prn, apr, pvd, pch, ofa, fit, rzx</i>	<i>phl, hcn, plt, prn, apr, pvd, pch, ofa, fit</i>	<i>phl, hcn, plt, prn, apr, pvd, pch, ofa, fit</i>	<i>phl, hcn, apr, pvd, pch, fit, rzx</i>	<i>phl, hcn, apr, pvd, pch, fit</i>
Plant protection efficacy ^a	Not tested in this study	++	++	++	+

* Paulsen et al. 2005.

** Jousset et al. 2014.

*** Takeuchi et al. 2014.

**** Takeuchi et al. 2015.

^a Plant protection efficacies were estimated as follows: ++, strong efficacy (gives more than the double weight of plants without pseudomonads); +, moderate efficacy (gives less than the double weight of plants without pseudomonads, but has a significant effect), as evaluated by measuring plant weights. Grouping was performed according to the honestly significant difference test. The index represents the average of 16 replicates (flasks containing three cucumber plants) per treatment with *Pythium ultimum* (modified from Takeuchi et al. 2014, 2015).

supported the PCR analysis results described above.

The reported genes associated with the Gac/Rsm signal transduction pathway were fully conserved in the genomes of Cab57, Os17, and St29, with >90% homology to those of Pf-5, suggesting that the components of the Gac/Rsm cascade are the core of each group of pseudomonads as an antibiotic producer. In strain Cab57, the defective mutant of RetS, which inhibits the activity of the Gac/Rsm pathway, exhibited the up-regulated expression of sRNA and *AprA* and increased antibiotic activities toward the phytopathogens *P. ultimum* and *Fusarium oxysporum* (Takeuchi et al. 2014). In strain Os17, the defective mutant of GacA exhibited decreased levels of its antibiotics, rhizoxin analogs, as described below.

Other typical gene clusters encoding biocontrol factors were also examined. Gene clusters for the biosynthesis of pyoverdine (*pvd*) and enantio-pyochelin (*pch*), the products of which have been commonly reported in *P. protegens*, were conserved in the genomes of Cab57, Os17, and St29. The gene cluster for orfamides (*ofa*) was found in the genome of Cab57, but was not conserved in the genomes of Os17 and St29. Phenazine is an antibiotic produced by some *Pseudomonas* strains (excluding *P. protegens*). A homology search of the gene cluster over the entire genome suggested that the known pathways for the synthesis of phenazine may not be present in the Cab57, Os17, and St29 strains.

Among the 519 CDSs specifically shared by the genomes of Os17 and St29, we found some species-specific traits: the genes annotated to be involved in O-antigen, type VI secretion, fimbriae, and pili. These homologs were also absent from other strains of *P. protegens* Pf-5 and CHA0 at >60% identity, suggesting that they contribute to building up the uniqueness of Os17 and St29 and fall into the same species.

Identification of biocontrol factors specific to Os17 by a genomic approach

In order to search for genes that may account for the better biocontrol efficacy observed in strain Os17 than in St29 (Table), we focused on a comparative genome analysis between these two strains and also specified the 256 CDSs unique to strain Os17. The complete rhizoxin analog biosynthesis gene cluster (*rxz*, ca. 79 kb) found in the Os17 genome was absent in the St29 genome. In an *rxzB* mutant, which lacks the polyketide synthase essential for the production of rhizoxin analogs, growth inhibitory activity against fungal and oomycete pathogens and plant protection efficacy were lower than those of wild-type Os17 (Takeuchi et al. 2015), suggesting that rhizoxin analogs are important biocontrol factors of this strain.

Potential applications and future perspectives for genome-based studies in biocontrol research

Several pseudomonad strains have been increasingly marketed as biocontrol agents. Among them, the genome of *P. fluorescens* strain A506, which is sold as BlightBan® A506 for the suppression of the bacterial disease fire blight in pear and apple orchards in the USA and Canada, has been deciphered in a comparative genomic project on biocontrol pseudomonads (Loper et al. 2012), providing some distinct features of this strain. Furthermore, *P. chlororaphis* MA 342, which is used for seed coating to suppress seed-borne pathogens, and the products marketed as Cedomon® and Cerall® in Europe are known to be producers of rhizoxin analogs (Johansson & Wright 2003). Recent advances in genomics have revealed the importance of rhizoxin analogs as biocontrol factors in strains of *P. protegens* Pf-5 (Loper et al. 2008, 2016) and *Pseudomonas* sp. Os17 (Takeuchi et al. 2015). This genome-based approach will continuously contribute to our understanding of the mechanisms underlying disease control. Genomic data will also enable us to identify the strains and factors that i) improve the suppression of plant diseases caused by specific pathogens and ii) contribute to the adaptation of strains in each type of soil (with different pH, moisture levels, organic matter content, and minerals), thereby allowing for detailed management strategies for sustainable agriculture. Further development of genome mining tools will promote the level of predictions of the functions of genes.

Recent studies on genomics also revealed that the Gac/Rsm pathway has been conserved in *P. protegens* and its related strains, whereas secondary metabolites showed strain-specific diversity. In addition to the application of these beneficial strains themselves, systematic cultural control, which favors the functions of the strains by designing suitable chemical properties of the rhizosphere, may be an effective approach to improve biocontrol efficacy.

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