Rhizoxin Analogs Contribute to the Biocontrol Activity of a Newly Isolated *Pseudomonas* Strain

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Two strains of *Pseudomonas* sp., Os17 and St29, were newly isolated from the rhizosphere of rice and potato, respectively, by screening for 2,4-diacetylphloroglucinol producers. These strains were found to be the same species and were the closest to but different from *Pseudomonas protegens* among the sequenced pseudomonads, based on 16S ribosomal RNA gene and whole-genome analyses. Strain Os17 was as effective a biocontrol agent as reported for *P. protegens* Cab57, whereas strain St29 was less effective. The whole-genome sequences of these strains were obtained: the genomes are organized into a single circular chromosome with 6,885,464 bp, 63.5% G+C content, and 6,195 coding sequences for strain Os17; and with 6,833,117 bp, 63.3% G+C content, and 6,217 coding sequences for strain St29. Comparative genome analysis of these strains revealed that the complete rhizoxin analog biosynthesis gene cluster (approximately 79 kb) found in the Os17 genome was absent from the St29 genome. In an *rxnB* mutant, which lacks the polyketide synthase essential for the production of rhizoxin analogs, the growth inhibition activity against fungal and oomycete pathogens and the plant protection efficacy were attenuated compared with those of wild-type Os17. These findings suggest that rhizoxin analogs are important biocontrol factors of this strain.

Pseudomonas protegens and some other root-colonizing pseudomonads classified into the *P. fluorescens* group are effective biocontrol strains, which suppress plant diseases in the rhizosphere. *P. protegens* strains Pf-5 and CHA0 (previously called *P. fluorescens* Pf-5 and CHA0, respectively) have been used as model strains in studies on the biosynthesis of several extracellular enzymes, such as AprA protease, and sec-

ondary metabolites with antibiotic activity, such as 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), and pyoluteorin (PLT) (Haas and Keel 2003). The complete genome data of strains Pf-5 and CHA0 and other pseudomonads are available at the Pseudomonas.com website (Winsor et al. 2011) and provide detailed annotation data of these strains.

The expression of biocontrol factors depends on the Gac/Rsm signal transduction pathway (Lapouge et al. 2008). The Gac/Rsm cascade is initiated by the GacS/GacA two-component system. The GacS sensor kinase is known to be phosphorylated and activates the cognate GacA response regulator by phosphotransfer during slow growth and in the presence of unidentified signal molecules. In *P. protegens* CHA0, activated GacA promotes the transcription of noncoding small RNAs (sRNAs) termed RsmX, RsmY, and RsmZ. These sRNAs have high affinity for the RNA-binding protein RsmA and also for the RsmA paralog RsmE in strain CHA0 (Heeb et al. 2002; Kay et al. 2005; Reimmann et al. 2005; Valverde et al. 2003). The RsmA and RsmE proteins repress the translation of genes involved in secondary metabolism during trophophase. When RsmX, -Y, and -Z sRNAs are induced in idiophase, they relieve the translational repression of target genes by sequestering the RsmA and RsmE proteins, thereby allowing the synthesis of secondary metabolites (Haas and Défago 2005; Kay et al. 2005, 2006). Thus, mutants defective in the Gac/Rsm signal transduction pathway have a reduced ability to produce such biocontrol factors and to suppress plant diseases (Haas and Défago 2005; Kim et al. 2011). Two additional sensors, termed RetS and LadS, were shown to provide input into the Gac/Rsm pathway in *P. fluorescens* and *P. aeruginosa* (Goodman et al. 2009; Humair et al. 2009). RetS inhibits and LadS activates the activity of the Gac/Rsm pathway and, in this way, both sensors affect the expression of target genes and biocontrol factors (Humair et al. 2009). Krebs cycle intermediates and alarmones ppGpp (guanosine tetraphosphate) are also involved in this pathway via the expression of Rsm sRNAs (Takeuchi et al. 2009, 2012).

In terms of the effective and ecological application of biocontrol agents, screening for pseudomonads that benefit plants has led to advances in biocontrol research. Moreover, the complete genomic sequence of strain Pf-5 (Paulsen et al. 2005) has provided new insights into the molecular basis underlying the pseudomonad-mediated suppression of plant disease. For example, novel natural products, including the cyclic lipopeptide orfamide A (Gross et al. 2007), rhizoxin analogs (Loper et al. 2008), and the insect toxin FitD (Péchy-Tarr et al. 2008), have been discovered through genomics-guided approaches in strains

The complete genome sequences of *Pseudomonas* sp. strains Os17 and St29 have been deposited in the DDBJ/EMBL/GenBank database under accession numbers AP014627 and AP014628, respectively.

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Pf-5 and CHA0. Comparative genome analysis among strains in the *P. fluorescens* group has also contributed to the understanding of other strains (Loper et al. 2012). In our previous work, we isolated and fully sequenced biocontrol strain *P. protegens* Cab57, and compared its genome with those of Pf-5 and CHA0. Even within the same species (*P. protegens*), comparative genome analysis revealed that each strain builds up strain-specific genomic repertoires for the biosynthesis of secondary metabolites and niche adaptation (Takeuchi et al. 2014).

In the present study, we aimed to screen biocontrol pseudomonad strains close to but different from *P. protegens* Cab57, with the expectation that their comparative genome analyses would contribute to the identification of factors involved in the biocontrol efficacy of newly isolated strains. We showed that two of the isolates producing DAPG exhibited different biocontrol potential against *Pythium* damping-off and root rot in the cucumber. We named the effective biocontrol isolate Os17 and the other one St29, and conducted whole-genome sequencing on them. A whole-genome comparison of these related strains revealed that they are the same species and they have several strain-specific genomic regions. Among these regions, the complete rhizoxin analog biosynthesis gene cluster (approximately 79 kb) specific to the Os17 genome was characterized in detail for its role in biocontrol activity of this strain.

RESULTS AND DISCUSSION

Isolation of DAPG-producing pseudomonads closely related to but different from reported strains of *P. protegens*.

Approximately 2,800 fluorescent pseudomonads were obtained from plant roots. On the basis of polymerase chain reaction (PCR) analysis of this collection to identify the *phlD*⁺ strain, 48 isolates were selected as candidates of DAPG-producing isolates and their production activities were confirmed by thin-layer chromatography (TLC) (data not shown). Of the 48 strains, five were PCR positive for all of the three other antibiotic biosynthetic genes (for PRN, PLT, and hydrogen cyanide [HCN]) that are typically found in *P. protegens* (Takeuchi et al. 2014). Actually, one of these five strains, Cab57, was identified as *P. protegens* based on 16S ribosomal RNA (rRNA) gene analysis (with 100% identity) and whole-genome analysis (Takeuchi et al. 2014). Among the remaining

strains, 39 strains were PCR positive for the *hcn* gene but negative for PRN and *plt* genes. On the basis of 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, 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 analysis. 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. For the detection of antibiotic activity, strains were grown on a nutrient broth supplemented with 1% glucose (NBglu), which is conducive to DAPG production in *P. fluorescens* Pf-5 (Kidarsa et al. 2011). The growth inhibition activities of strains Os17 and St29 against *Bacillus subtilis* on an NBglu agar plate were assessed by the halo size, giving values of 13.5 ± 0 and 13.3 ± 0.2 mm, respectively. DAPG production levels by these strains were determined by high-performance liquid chromatography (HPLC). The concentrations of DAPG were 498 ± 95 nmol and 207 ± 13 nmol, respectively, per optical density at 600 nm (OD_{600}) in the strains grown in liquid NBglu to $OD_{600} = 4.0$ (one OD_{600} unit corresponds to approximately 1×10^9 cells/ml).

Biocontrol efficacy of strains Os17 and St29.

To investigate the biocontrol activity of strains Os17 and St29 in a natural habitat, we adopted a cucumber–*Pythium ultimum* pathosystem, which enabled us to evaluate plant protection efficacy by measuring the root and shoot weights. Strain Os17 was as effective a biocontrol agent as reported for strain Cab57, whereas strain St29 was less effective than strain Os17, with 95% confidence (Table 1).

The genome structures of Os17 and St29.

Although DAPG is one of the most important antimicrobial compounds produced by biocontrol strains of the *Pseudomonas fluorescens* group, there are many other antibiotic metabolites, some of which have been demonstrated to be involved in their biocontrol activity. Furthermore, other bacterial traits such as siderophore production and motility are also important

Table 1. Suppression of *Pythium* damping-off and root rot in the cucumber by *Pseudomonas protegens* Cab57, *Pseudomonas* sp. Os17, and *Pseudomonas* sp. St29

Bacterial strain added ^a	<i>Pythium</i> added ^a	Per flask ^x		Root fresh weight per flask (g) ^y	
		Surviving plants (%)	Shoot fresh weight (g)	1st Exp.	2nd Exp.
None	–	100 a	0.71 a	0.36 a	0.30 a
Cab57	–	100 a	0.73 a	0.37 a	0.29 a
Os17	–	100 a	0.68 a	0.38 a	0.31 a
St29	–	100 a	0.72 a	0.42 a	0.31 a
None	+	6 c	0.21 d	0.11 d	0.10 d
Cab57	+	75 a	0.45 bc	0.21 c	0.19 bc
Os17	+	83 a	0.47 b	0.27 b	0.21 b
St29	+	50 b	0.39 c	0.17 cd	0.15 cd

^x Data represent the averages of 10 replicates (flasks containing three cucumber plants) per treatment without *Pythium ultimum* and 16 replicates per treatment with *P. ultimum*. Means within the same column followed by different letters (a–d) are significantly different ($P < 0.05$) according to Tukey's honestly significant difference (HSD) test.

^y Data represent the averages of five replicates (flasks containing three cucumber plants) per treatment without *P. ultimum* and eight replicates per treatment with *P. ultimum* in each experiment. Means within the same column followed by different letters (a–d) are significantly different ($P < 0.05$) according to Tukey's HSD test.

^z *Pseudomonas* strains were added at 10^7 CFU/g of vermiculite contained within 100-ml flasks (30 g of soil per flask), after planting three 92-h-old, sterile-grown cucumber seedlings per flask. *P. ultimum* was added as a millet-seed inoculum at 2.5 g/kg of vermiculite before planting. Plants were harvested after 7 days.

for efficient plant protection (Haas and Keel 2003). Therefore, the higher efficiency of DAPG production in strain Os17 may not solely account for the advantage in biocontrol activity. To obtain overviews of functionality and features of the newly isolated biocontrol strain Os17, we conducted whole-genome sequencing. We also obtained the whole-genome data of strain St29, enabling comparison between two strains. The genomes are organized into a single circular chromosome with 6,885,464 bp and 63.5% G+C content for strain Os17, and with 6,833,117 bp and 63.3% G+C content for strain St29, and the genomes are predicted to encode 6,195 and 6,217 proteins, respectively. The general genomic features of strains Os17 and St29 as well as those of *P. protegens* Cab57 are listed in Table 2. Strains Os17 and St29 were deposited in the MAFF Genebank, National Institute of Agrobiological Sciences, as MAFF212089 and MAFF212090, respectively.

To define species, the JSpecies program has been commonly used to compare two genomes (Richter and Rosselló-Móra 2009); thus, we applied this program for the species identification of strain Os17 and strain St29. The average nucleotide identity, calculated with BLAST algorithm (ANiB) values, which provides a numerical and stable species boundary (Richter and Rosselló-Móra 2009), confirmed the categorization of strains Os17 and St29 into the same species (98.81%) (Table 3); values higher than 96% were found for strains of the same species. On the other hand, the values were lower than 90% when compared with the genome of strain Os17 and those of *P. protegens* strains (Cab57, CHA0, and Pf-5). Together with ANiB, tetranucleotide frequency correlation coefficients (TETRA) reinforced the objective boundary for species circumscription when TETRA values >0.99; however, both values should agree (Richter and Rosselló-Móra 2009). Comparison of the genomes of strains Os17 and St29 showed a value of >0.999 (Table 3). We also performed comparisons with genomes of other *P. fluorescens* group strains: *P. chlororaphis* subsp. *aureofaciens* 30-84, *P. fluorescens* Pf0-1, *P. fluorescens* SBW25, and *P. fluorescens* A506, covering all three of the subclades of the *P. fluorescens* group, in which the strains are divided as follows: group 1 = *P. protegens* Pf-5, *P. chlororaphis* subsp. *aureofaciens* strains 30-84, and O6; group 2 = *P. fluorescens* strains Pf0-1 and Q2-87 and *P. brassicacearum* Q8r1-96; and group 3 = *P. fluorescens* strains SS101, A506, and SBW25 and *P. synxantha* BG33R (Loper et al. 2012). ANiB values were lower than those of *P. protegens* strains, suggesting that strains Os17 and St29 are the closest relatives to *P. protegens* among the studied strains, although they are not identified as *P. protegens*.

Then, we constructed a whole-genome-based phylogenetic tree to illustrate the taxonomic position of strains Os17 and St29 among other *P. fluorescens* group strains by using a reference sequence alignment-based phylogeny builder (REAL-

PHY) (Bertels et al. 2014). These two strains fall into the same subtree, creating a new branch within group 1 (Supplementary Fig. S1), and these are the closest relatives to strains of *P. protegens*, supporting the results of the species definition mentioned above. On the basis of this method, *P. fluorescens* group strains were separated into three subclades, consistent with the report by Loper and associates (2012), in which the tree was based on concatenated alignments of 10 core house-keeping genes.

Core and pan-genome analysis.

A BLASTp search of strains Os17, St29, and Cab57 revealed that unique coding sequences (CDS) were most abundant in strain Cab57 (Fig. 1), reflecting its heterology among the three strains. The number of Cab57-specific CDS was 736, whereas it was 234 when comparing the genome with those of the strains of *P. protegens* Pf-5 and CHA0 (Takeuchi et al. 2014). Strains Os17 and St29 shared 519 CDS, except for core genes (5,321), some of which may reflect the species-specific repertoire, as mentioned below. Strain Cab57 shared more CDS with strain Os17 than with strain St29 (Fig. 1) and, of the 99 CDS shared specifically by Cab57 and Os17, 55 were phage-related genes, as also mentioned below.

Concerning biocontrol factors, two gene clusters (*hcn* and *phl*) encoding antibiotic metabolites were conserved in the genomic sequence of strains Os17 (gene ID 2580-2582 and 5893-5900) and St29 (gene ID 2684-2686 and 5899-5906), whereas two other gene clusters (*plt* and *prn*) were absent, which supported the PCR analysis results mentioned above. The *aprA* gene cluster, for the major extracellular protease AprA, is conserved in the two strains (gene ID 2865-2870 for Os17 and gene ID 2982-2988 for St29), although a homolog of the *aprE* gene presents as a hybrid of two adjacent genes (2986 and 2987) in St29 (Supplementary Table S1).

The reported genes associated with the Gac/Rsm signal transduction pathway were fully conserved in the Os17 genome, with >90% homology to those of Pf-5 (Supplementary Table S2). All of these homologs of Os17 were completely conserved in the St29 genome, with 100% homology (data not shown), suggesting that the components of the Gac/Rsm cascade are the core of each group of pseudomonads as an antibiotic producer.

Other typical gene clusters encoding biocontrol factors were also explored (Supplementary Table S3). Gene clusters for pyoverdine (*pvd*), whose product has been commonly reported in *P. protegens*, were conserved in the Os17 genome in four different loci (gene ID 3176-3177, 4178-4197, 4256-4266, and 4277-4279) as reported in Pf-5 (Gross and Loper 2009) and Cab57 (Takeuchi et al. 2014), although genes of 4191 to 4195 showed lower homologies (40 to 53% identity) to these strains. The gene cluster for enantio-pyochelin (*pch*), which has been

Table 2. General genomic features of *Pseudomonas protegens* Cab57, *Pseudomonas* sp. Os17, and *Pseudomonas* sp. St29

Strains	Genome size (bp)	CDS number	G+C content (%)	rRNA genes number	tRNA genes number	Accession number
Cab57	6,827,892	6,186	63.3	16	68	AP014522
Os17	6,885,464	6,195	63.5	19	70	AP014627
St29	6,833,117	6,217	63.3	19	68	AP014628

Table 3. Whole-genome comparison of strain Os17 with those of St29 and other *Pseudomonas fluorescens* group

Comparison ^z	Strains within the <i>P. fluorescens</i> group						
	St29	Cab57	CHA0	Pf-5	Pf0-1	SBW25	A506
ANiB (%)	98.81	89.72	89.63	89.45	81.34	80.67	80.41
TETRA value	0.99987	0.9953	0.99518	0.99524	0.96023	0.94454	0.94047

^z ANiB = average nucleotide identity, calculated with BLAST algorithm, and TETRA = tetranucleotide frequency correlation coefficients to Os17.

isolated from strain CHA0 and its gene cluster and is fully conserved in Pf-5 (Youard et al. 2007), was also conserved in strain Os17. Both *pvd* and *pch* clusters were conserved in strain St29.

The gene cluster for orfamides, which has been identified in strain Pf-5 from mining of *Pseudomonas* genomes (Gross et al. 2007) and also found in the genomes of CHA0 and Cab57 (Takeuchi et al. 2014), was not conserved in the two genomes of Os17 and St29. Phenazine is an antibiotic produced by some *Pseudomonas* strains (excluding *P. protegens*). The 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 both Os17 and St29 strains.

Among the 519 CDS specifically shared by the genomes of Os17 and St29 (Fig. 1), we listed regions including at least four continuous open reading frames (ORFs) in order to obtain an overview of species-specific traits (Supplementary Table S4, clusters OS1 to OS33). Some of these clusters should be mentioned, as follows: OS1 for O-antigen, OS7 for type VI secretion, OS25 for fimbriae, OS26 for secretion, and OS29 for pilus. These homologs were also absent from other strains of *P. protegens* Pf-5 and CHA0 at >60% identity, suggesting that they may contribute to build up the uniqueness of Os17 and St29 and fall into the same species. OS3, OS16, and OS21 were identified as parts of secondary metabolite clusters using the antiSMASH program (Blin et al. 2013). According to this program, OS3 was predicted to encode bacteriocin. OS16 (located at 3369090..3376837 in the Os17 genome) was part of a large hit region (3266543..3385308), including *rxz* cluster (3285485..3365308), although the relevance of this is not clear. OS21 (located at 4152719..4168145 in the Os17 genome) was also part of a large hit region (4111697..4163109), including mitomycin clusters O6 (4111262..4130682) and O7 (4131697..4152441).

Fig. 1. Venn diagram comparing the coding sequence sets of *Pseudomonas* strains Os17 and St29 and *Pseudomonas protegens* Cab57. The number of orthologous coding sequences (CDS) shared by all strains is in the center. Numbers in the nonoverlapping portions of each circle represent the number of CDS unique to each strain. The total number of CDS within each genome is listed below the strain name. Each set of genes was compared with the other two sets using BLASTp, and sequence identity cut-off was set at 60% to identify common genes. Note that, owing to multiple hits, gene numbers do not add up to the strain totals.

The rhizoxin analog biosynthesis gene cluster in the Os17 genome, which was absent from the St29 genome.

We performed dot plot analysis to obtain an overview of chromosome-scale genomic synteny among the strains of Os17, St29, and *P. protegens* Cab57 (Fig. 2). Compared with the Os17 genome, two large segments were absent from the St29 genome (Fig. 2A). One of these segments was a homolog of the complete *rxz* gene cluster (approximately 79 kb) (Supplementary Fig. S2), which has been reported to encode genes for the biosynthesis of analogs of the antimetabolic macrolide rhizoxin in *P. protegens* Pf-5 (Loper et al. 2008). This cluster has been found to be absent from two other fully sequenced *P. protegens* strains, CHA0 and Cab57 (Takeuchi et al. 2014). In addition, another large segment was phage-related elements, as mentioned below. When comparing the genome of Os17 with that of Cab57, two large inversions were found (Fig. 2B). These two inversions were also found in the genome of St29 (data not shown).

At the upstream region of the *rxz* gene cluster, the fit cluster encoding a functional insect toxin reported in *P. protegens*

Fig. 2. Syntenic dot plot of *Pseudomonas* strain. Dot plot comparison of **A**, the Os17 genome versus the St29 genome and **B**, the Os17 genome versus the *P. protegens* Cab57 genome. The line represents the regions of similarities between the two genomes, while discontinuities in this syntenic line represent regions of genomic variations at a given locus between the two strains. The position of the biosynthetic gene cluster for rhizoxin analogs in the Os17 genome is indicated by arrows (*rxz* in A and B). Positions of phage-related elements are also marked by an arrow (prophage in A). Genomic inversions observed between the genomes of Os17 and Cab57 are marked by horizontal arrows in B.

CHA0 (Péchy-Tarr et al. 2008) was found to be conserved in the Os17 genome. This cluster is also conserved in the St29 genome.

Regions unique to the Os17 genome.

Apart from the rhizoxin analog biosynthesis gene cluster unique to the Os17 genome, another large segment found in dot plot analysis with the St29 genome was the region for phage-related elements (Fig. 2). This segment corresponds to the gene ID 3421-3565 of the Os17 genome and the gene ID 3520-3546 of the St29 genome, both of which consist of regions unique to each other. Gene ID 3421-3565 of the Os17 genome showed similarity to Cab57 prophages III and IV over the region and partly homologous to Cab57 prophage I, which have been previously reported in the Cab57 genome (Takeuchi et al. 2014). Gene ID 3520-3546 of the St29 genome contains five genes for transposase, suggesting that this region is relevant to transposons, and it also contains six genes involved in pilus assembly. This transposon-like region was absent from the Cab57 genome.

As other large regions unique to the Os17 genome, apart from the *rxz* gene cluster, we listed regions including at least four continuous ORFs missing in the St29 and Cab57 genomes (Supplementary Table S5, clusters O1 to O9). Among these

clusters, clusters O5 (gene ID 3675-3692) and O6 (gene ID 3695-3712) caught our attention because these were identified as secondary metabolite clusters using the antiSMASH program. These two neighboring clusters are homologs of the biosynthetic cluster for the antibiotic mitomycin from *Streptomyces lavendulae* (Mao et al. 1999). Although the role of mitomycin in biocontrol has not been elucidated, such gene clusters may contribute to niche adaptation by this strain from an ecological viewpoint.

Construction of the *rxzB* mutant and its effect on rhizoxin analog production.

Strain specificity of the complete *rxz* gene cluster in the genome of Os17 among the three strains compared (Os17, St29, and Cab57) prompts us to predict that this region enables the strain to be superior to St29 as a biocontrol strain, and that it may compensate for the absence of *plt* and *prn* to exert its biocontrol activity at a level similar to that of strain Cab57. In a previous study, naturally isolated strains of *Pseudomonas* spp. that produce rhizoxin analog were shown to suppress phytopathogenic fungi and an oomycete in culture (Johansson and Wright 2003). Furthermore, the growth inhibition activities of

Fig. 3. Expression of an *rxzB*'-*lacZ* fusion on pME6014*rxzB* and an *rxzI*'-*lacZ* fusion on pME6014*rxzI* in Os17 and the *gacA* mutant grown in nutrient yeast broth. Symbols indicate averages of triplicate cultures and bars indicate standard deviations. OD_{600nm} = optical density at 600 nm.

Fig. 4. Effects of the *rxzB* mutation on antibiotic activity. Antibiotic activities of *Pseudomonas* sp. Os17 strains toward **A**, *Pythium ultimum* and **B**, *Fusarium oxysporum* were evaluated by the size of the growth inhibition zone. Antibiotic activities of Os17 wild type (WT) and the *rxzB* mutant (Δ *rxzB*) were compared.

rhizoxin and its analogous compounds toward fungus have been demonstrated (Iwasaki et al. 1984; Loper et al. 2008). Nevertheless, their role in biocontrol activity has not been assessed. To investigate the role of rhizoxin analogs in strain Os17, we constructed a rhizoxin-analog-negative mutant by knocking out the *rxzB* gene, the first gene in the rhizoxin gene cluster. In *P. protegens* Pf-5, *rxzB* had been annotated as polyketide synthase and reported to be essential for the production of rhizoxin analogs (Loper et al. 2008). The levels of production of the reported rhizoxin analogs (Rhizoxin, Rhizoxin D, WF-1360B, WF-1360C, WF-1360F, and 22Z-WF-1360F) were determined by liquid-chromatography mass spectrometry (LC-MS). In contrast to the wild type (Supplementary Fig. S3), accumulation of rhizoxin analogs in the *rxzB* mutant was below the detection limit (Supplementary Fig. S4). The wild type produced at least five rhizoxin analogs with $[M + H]^+$ peaks at 580, 594, 596, 610, and 626 m/z for WF-1360C, Rhizoxin D, WF-1360B, 22Z-WF-1360F or WF-1360F, and Rhizoxin, respectively. At the m/z of 594 and 626, multiple peaks were detected, suggesting the presence of isomers for each analog.

A *gacA* mutant exhibits decreased expression of *rxzB'*-*lacZ* and *rxzI'*-*lacZ* fusions.

In *P. protegens* and other fluorescent pseudomonads, the GacS/GacA system positively controls the secondary metabolism. The production of rhizoxin analogs has also been reported to be under the control of GacA in *P. protegens* Pf-5, showing that, in the *gacA* mutant, the production was abolished and the levels of transcripts of two genes (PFL_2988 and PFL_2991) within the rhizoxin biosynthetic cluster were lower than those of the wild type (Hassan et al. 2010).

To determine whether the production of rhizoxin analogs in strain OS17 is under the control of the GacS/GacA system as well, we constructed a *gacA* mutant and determined the production levels of rhizoxin analogs by LC-MS. In the *gacA* mutant, the accumulation of rhizoxin analogs was below the detection limit (Supplementary Fig. S5).

In the Gac/Rsm signal transduction pathway of *P. protegens* CHA0, the RsmE (or RsmA) protein makes optimal contacts with an RNA sequence, 5'- Δ /CANGGANG Δ /3', in which the central ribonucleotide forms a hexaloop (Shubert et al. 2007). In the rhizoxin biosynthetic cluster of the Os17 genome, the *rxzB* gene (gene ID 2970, homolog of PFL_2989 in Pf-5) and the *rxzI* gene (gene ID 2976, homolog of PFL_2995 in Pf-5) have this consensus sequence in their upstream region. Therefore, we constructed translational *rxzB'*-*lacZ* fusion and *rxzI'*-*lacZ* fusion, and compared their expression in the wild

type and the *gacA* mutant (Fig. 3; Supplementary Fig. S6). In the *gacA* mutant, a decreased level of expression was observed in both fusions at high cell densities, suggesting that GacA-dependent regulation of these genes may account, at least in part, for the decreased level of rhizoxin analogs in the *gacA* mutant. Furthermore, the *gacA* mutant loses its antibiotic activity against *B. subtilis*, as reported in other biocontrol strains of pseudomonads (data not shown).

An *rxzB* mutant exhibited decreased antibiotic activity and biocontrol efficacy.

We investigated whether the production of these compounds would affect the biocontrol efficacy in strain Os17 by utilizing the *rxzB* mutant. In the *rxzB* mutant, the growth inhibition activities against the phytopathogenic fungus *Fusarium oxysporum* and oomycete *Pythium ultimum* were attenuated (Fig. 4). These results indicate that the production of rhizoxin analogs may influence the biocontrol capacity of strain Os17. To investigate the influence of the *rxzB* mutation in soil, we used a cucumber-*P. ultimum* pathosystem. In terms of biocontrol efficacy, the *rxzB* mutant was less effective than the wild type (with 95% confidence for survival test and shoot weight, and with 90% confidence for root weight) (Table 4). Both the wild type and the mutant reached similar levels of root colonization (Table 4).

In conclusion, comparative genome analysis of two newly isolated *Pseudomonas* strains was efficient to identify important biocontrol factors and to obtain an overview of traits of each strain. Among the regions specific to a more effective biocontrol strain, the complete rhizoxin analog biosynthesis gene cluster was characterized in detail for its role in biocontrol activity. Rhizoxin was first isolated from *Rhizopus chinenensis*, a pathogenic fungus causing rice seedling blight with a symptom of abnormal swelling of roots, as its virulence factor (Iwasaki et al. 1984). Rhizoxin was then found to be produced by *Burkholderia rhizoxinica*, the bacterial endosymbiont of the fungus, but not by *Rhizopus* (Partida-Martinez and Hertweck 2005). The rhizoxin biosynthesis gene cluster (*rhi* gene cluster) was reported in *B. rhizoxinica* (Partida-Martinez and Hertweck 2007), and its whole-genome sequencing revealed that this cluster is encoded on the chromosome but not the megaplasmid carried by this strain (Lackner et al. 2011). The high conservation of this cluster between distant species (*Pseudomonas protegens* Pf-5 or *Pseudomonas* sp. Os17 and *B. rhizoxinica*) suggests the evolutionary traces of horizontal gene transfer, although its mechanism is yet to be elucidated. Study of the distribution of this gene cluster in rhizosphere bacteria may provide intriguing insights into the interactions among benefi-

Table 4. Effects of *rxzB* mutation on the suppression of *Pythium* damping-off and root rot of cucumber by *Pseudomonas* sp. strain Os17

Bacterial strain added ^w	<i>Pythium</i> added ^w	Surviving plants per flask (%) ^x	Shoot fresh weight per flask (g) ^x	Root fresh weight per flask (g) ^y	Colonization by <i>Pseudomonas</i> (log ₁₀ CFU/g of root) ^z
None	–	100 a	0.71 a	0.30 a	ND
Os17 (wild type)	–	100 a	0.65 a	0.29 a	7.06 ± 0.13
Δ <i>rxzB</i>	–	100 a	0.70 a	0.30 a	6.88 ± 0.17
None	+	2 c	0.18 d	0.08 d	ND
Os17 (wild type)	+	85 a	0.43 b	0.18 b	8.25 ± 0.21
Δ <i>rxzB</i>	+	58 b	0.35 c	0.15 c	8.43 ± 0.28

^w *Pseudomonas* strains were added at 10⁷ CFU/g of vermiculite contained within 100-ml flasks (30 g of soil per flask), after planting three 92-h-old, sterile-grown cucumber seedlings per flask. *P. ultimum* was added as a millet-seed inoculum at 2.5 g/kg vermiculite before planting. Plants were harvested after 7 days.

^x Data represent the averages of 10 replicates (flasks containing three cucumber plants) per treatment without *P. ultimum* and 16 replicates per treatment with *P. ultimum*. Means within the same column followed by different letters (a to d) are significantly different ($P < 0.05$) according to Tukey's honestly significant difference (HSD) test.

^y Data represent the averages of 10 replicates (flasks containing three cucumber plants) per treatment without *P. ultimum* and 16 replicates per treatment with *P. ultimum*. Means within the same column followed by different letters (a–d) are significantly different ($P < 0.1$) according to Tukey's HSD test.

^z The rhizosphere-stable plasmid pME6031 containing a tetracycline resistance determinant (Heeb et al. 2000) was introduced as a selective marker into the bacterial strains to determine their root colonization capacity in soil. ND = not detected.

cial and phytopathogenic microbes and plants. Considering the metabolic load and the cost of harboring such a large cluster in the genome, it can be speculated that the *rxz* cluster does not necessarily make the strain effective under conditions in which a simple lifestyle is preferable for survival. Indeed, there are many other biocontrol factors such as DAPG and HCN whose gene clusters are more common among biocontrol pseudomonad strains. The fact that Os17 was isolated from the rhizosphere of rice tempts us to speculate on whether the host plant species is relevant to the strain-specific genomic repertoires for the biosynthesis of secondary metabolites. Investigation of the interactions between rhizoxin analogs producing pseudomonads and host rice is a challenge for our future work.

Comparative genomics between closely related strains revealed phenotype-specific genes and enabled us to identify strain-specific biocontrol factors. In addition to the *rxz* cluster, other Os17-specific gene clusters involved in secondary metabolites are also intriguing for our future study. Each repertoire of biocontrol factors may contribute to different potentials for niche adaptation in each strain.

MATERIALS AND METHODS

Isolation of DAPG-producing pseudomonads from rhizosphere soil.

For the isolation of fluorescent pseudomonads, plant roots were sampled on private lands in diverse locations (Hokkaido, Ibaraki, Kanagawa, Nagano, Aichi, and Nagasaki) in Japan after the land owners gave permission to conduct sampling on the sites. None of these plants is an endangered or protected species.

We isolated fluorescent pseudomonads based on a method described previously (Someya et al. 2012). DAPG-producing isolates were prescreened by PCR using the DAPG biosynthesis gene *phlD*-specific primers Phl2a and Phl2b. *phlD*⁺ isolates were incubated on dNBYG medium (Duffy and Dfago 1999) and DAPG production was evaluated by TLC (Keel et al. 1992). DAPG production was also evaluated in strains Os17 and St29 by HPLC (Schnider-Keel et al. 2000). Other antibiotic biosynthetic genes were also explored by PCR analysis using specific sets of primers: PRND1/PRND2 for PRN, PltBf/PltBr for PLT, and PM2/PM7-26R for HCN. Sequence

analysis of 16S rRNA genes was conducted as described previously (Someya et al. 2012).

Bacterial strains and growth conditions.

The bacterial strains and plasmids used are listed in Table 5. *Escherichia coli* and *Pseudomonas* strains were routinely grown in nutrient yeast broth (NYB) (2.5% [wt/vol] nutrient broth and 0.5% [wt/vol] yeast extract) and Luria-Bertani medium with shaking, or on nutrient agar plates (4% [wt/vol] blood agar base and 0.5% [wt/vol] yeast extract) amended with the following antibiotics, when required: ampicillin, 100 µg/ml; kanamycin, 25 µg/ml; or tetracycline, 50 µg/ml (100 µg/ml for selection of *Pseudomonas* sp.). The inoculation temperatures were 30°C for *Pseudomonas* sp. and 37°C for *E. coli*. *Pseudomonas* sp. was grown in Davis minimal broth without dextrose containing 20 mM glycerol (Davis-gly), which offers higher expression of *rxzB* in *P. protegens* Pf-5 (Loper et al. 2008) for the assays concerning rhizoxin analogs.

DNA manipulation.

Small-scale plasmid extraction was performed with a QIAprep spin miniprep kit (Qiagen); large-scale preparations were obtained with a Qiagen plasmid midi kit (Qiagen). Chromosomal DNA from *P. protegens* was prepared with a Qiagen Genomic DNA buffer set and Genomic-tip (Qiagen). DNA fragments were purified from agarose gels with a QIAquick gel extraction kit (Qiagen). Oligonucleotides used are listed in Supplementary Table S6.

Whole-genome sequencing and assembly.

Whole-genome sequencing was performed using Roche Genome Sequencer FLX+ technology provided by Eurofins Genomics K.K. (Tokyo). A shotgun library was prepared and subsequently sequenced, generating 322,380 reads in 154.0 Mbp of sequencing data for Os17 and 322,577 reads in 191.5 Mbp of sequencing data for St29. An 8-kb-long paired-end library was also prepared and sequenced, generating 363,796 reads in 60.6 Mbp of sequencing data for Os17 and 301,760 reads in 45.4 Mbp of sequencing data for St29. Co-assembly of the results of both shotgun and paired-end sequencing was performed using Newbler 2.6, with full assembly into 43 (for Os17) and 38 (for St29) large contigs, defined as > 1kb. These

Table 5. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^z	Source or reference
Strains		
<i>Bacillus subtilis</i>		
M168	Wild type	C. Keel
<i>Escherichia coli</i>		
DH5��, HB101	Laboratory strains	Sambrook et al. 2001
<i>Pseudomonas protegens</i>		
Cab57	Wild type	Takeuchi et al. 2014
<i>Pseudomonas</i> sp.		
Os17	Wild type	This study
Os17gacA	��gacA	This study
Os17rxzB	��rxzB	This study
St29	Wild type	This study
Plasmids		
pCR-Blunt II-TOPO	Cloning vector, pUC ori; Km ^r	Invitrogen
pME497	Mobilizing plasmid, IncP-1, Tra, RepA(Ts); Ap ^r	Voisard et al. 1988
pME3087	Suicide vector, ColE1 replicon, Mob; Tc ^r	Voisard et al. 1994
pME3087gacA	pME3087 containing a <i>Bam</i> HI/ <i>Hind</i> III 1.5-kb <i>gacA</i> region containing a deletion of the <i>gacA</i> gene; Tc ^r	This study
pME3087rxzB	pME3087 containing a <i>Bam</i> HI/ <i>Hind</i> III 1.6-kb <i>rxzB</i> region containing a deletion of the <i>rxzB</i> gene; Tc ^r	This study
pME6014	Cloning vector for construction of translational ' <i>lacZ</i> ' fusions; Tc ^r	Blumer et al. 1999
pME6014rxzB	Translational <i>rxzB</i> '-' <i>lacZ</i> fusion under <i>ptac</i> ; Tc ^r	This study
pME6014rxzI	Translational <i>rxzI</i> '-' <i>lacZ</i> fusion under <i>ptac</i> ; Tc ^r	This study
pME6031	pACYC177-pVS1 shuttle vector; Tc ^r	Heeb et al. 2000

^z Km^r, Ap^r, and Tc^r = resistant to kanamycin, ampicillin, and tetracycline, respectively.

contigs could be manually integrated into only one scaffold for each organism using BLAST and the Staden software package. The sizes of the resulting scaffolds were 6.89 Mbp for Os17 and 6.83 Mbp for St29. The scaffolds did not contain gaps. The prediction of putative coding sequences and gene annotation was performed using the Microbial Genome Annotation Pipeline.

Accession numbers.

The complete genome sequences of *Pseudomonas* sp. Os17 and *Pseudomonas* sp. St29 have been deposited in the DDBJ/EMBL/GenBank database under accession numbers AP014627 and AP014628, respectively.

Species identification and phylogenetic tree construction.

To identify species, whole-genome comparisons of strain Os17 or St29 with those of the *P. fluorescens* group were performed using the JSpecies program (Richter and Rosselló-Móra 2009). This program is commonly used to compare two genomes and uses BLAST software (Altschul et al. 1990). A phylogenetic tree was constructed using REALPHY (Bertels et al. 2014) with whole genomes of sequenced *Pseudomonas* species, applying the Pf-5 genome as a reference.

Comparative genome analysis.

To compare the whole genomes of *Pseudomonas* sp. Os17, *Pseudomonas* sp. St29, and *P. protegens* Cab57, the alignment data between each pair of strains' genome sequences were prepared using the LASTZ program (release 1.02.00), and dot plots were then produced from the alignment data using R (version 3.0.1). Genomic regions that were unique to each strain were searched for using the Island Viewer program (Langille et al. 2010) and verified manually. Secondary metabolite production clusters were examined using the antiSMASH program (Blin et al. 2013).

Plant disease suppression assays.

Biocontrol activity of each strain was evaluated as described previously (Takeuchi et al. 2012), with slight modifications. Flasks containing 30 g of vermiculite were planted with three cucumber seedlings each and treated with *Pythium ultimum* MAFF425494. *Pseudomonas* strains were added to soil as a suspension (3 ml per flask) of cells washed twice in sterile distilled water to give 1×10^7 CFU per g of soil. Control flasks received the same amount of sterile water. Seedlings were covered with 5 g of untreated vermiculite and flasks were sealed with aerated silicon caps. The microcosms were incubated in a growth chamber at 60% relative humidity and 26°C with light for 16 h, followed by an 8-h dark period. No watering was necessary. After 7 days of incubation, the biocontrol activity and root colonization of each strain were assessed. Data in Tables 1 and 4 represent the means from two individual repetitions of the same experiment, except for the result of root weight in Table 1, which represents the result of each experiment. Data of both experiments were first analyzed for a trial by treatment interaction by analysis of variance, which indicated that the data from the two independent trials could be pooled, except for the result of root weight in Table 1. Means were separated using Tukey's honestly significant difference test (at $P \leq 0.05$, except for the root weight in Table 4, for which $P \leq 0.1$ was used). Data for CFU counts were $\log_{10}$ -transformed prior to statistical analysis. Statistical analysis was performed using R (version 3.0.1).

Generation of *gacA*- and *rxzB*-negative mutant.

A deletion of the chromosomal *gacA* gene and a not-in-frame deletion in the chromosomal *rxzB* gene of *Pseudomonas*

sp. Os17 were created as follows. For *gacA*, fragments of 750 bp located upstream and 740 bp located downstream of the *gacA* gene were amplified by PCR with the primer pairs GacAUF/GacAUR and GacADF/GacADR, respectively. For *rxzB*, fragments of 790 bp located at positions 6,790 to 7,580 of the *rxzB* gene and 780 bp located at positions 9,400 to 10,180 of the *rxzB* gene were amplified by PCR with the primer pairs RxzBUF/RzxBUR and RxzBDF/RzxBDR, respectively. For these amplifications, high-fidelity DNA polymerase KOD Plus (Toyobo) and genomic DNA of *Pseudomonas* sp. Os17 as a template were used.

Each of the two corresponding fragments was annealed and amplified as a 1.5- or 1.6-kb fragment using the primer pair GacAUF/GacADR or RxzBUF/RzxBDR, respectively. These 1.5- and 1.6-kb fragments were cloned into pCR-Blunt II-TOPO (Invitrogen). The obtained inserts were confirmed by sequencing and digested with *Bam*HI and *Hind*III. After sequencing, these fragments were subcloned into pME3087 cleaved at the *Bam*HI and *Hind*III sites to give pME3087gacA or pME3087rxzB. These plasmids were mobilized from *E. coli* DH5 α to *Pseudomonas* sp. Os17 by triparental mating with *E. coli* HB101/pME497. Excision of the vector via a second crossing-over was obtained after the enrichment of tetracycline-sensitive cells, generating the *gacA* or *rxzB* mutant.

Construction of *rxzB*'-'*lacZ* and *rxzI*'-'*lacZ* fusion.

A 360- or 210-bp *Eco*RI-*Bam*HI fragment from the upstream region of *rxzB* or *rxzI* was amplified using primer sets of RxzBproF/RxzBproR or RxzIproF/RxzIproR, and inserted into pME6014 to obtain pME6014rxzB or pME6014rxzI containing a translational *rxzB*'-'*lacZ* or *rxzI*'-'*lacZ* fusion, respectively.

Detection of rhizoxin analogs.

To detect the rhizoxin analogs, spent culture supernatant extracts of strain Os17 and the mutants were prepared. The strains were grown with shaking (160 rpm) in 0.5 liters of Davis-gly medium at 25°C. After 48 h of incubation, cells were collected (calculated as $OD_{600} \times$ sampling volume of culture in milliliters of approximately 600), and the supernatant was extracted with ethyl acetate and evaporated to dryness. The crude extracts were dissolved in 600 μ l of MeOH, and 10 μ l was evaluated by LC-MS using a 2-mM ammonium acetate-buffered MeOH/H₂O gradient, increasing the MeOH from 10 to 95% over 10 min and holding it at 100% MeOH for 10 min (Cadenza CD-C₁₈; 150 by 2.0 mm; 3- μ m column; 0.20 ml/min flow rate, with total ion current and photodiode array monitoring with a wavelength range of 190 to 800 nm). LC-MS measurements were performed using a Q Exactive LC-MS Mass Spectrometer with an electrospray ion source (Thermo Fisher Scientific). Preparative HPLC was performed on a Shimadzu Nexera HPLC system. The positive ion mass spectra were obtained in full ion-scan mode (scanning range m/z 100 to m/z 1,500) at a resolution of 140,000. LC-MS was performed by Toray Research Center Inc., Otsu, Japan.

β -Galactosidase assays.

β -Galactosidase activities were quantified by the Miller method (Miller 1972). *Pseudomonas protegens* strains were grown at 30°C in 50-ml flasks containing 15 ml of NYB supplemented with 0.05% Triton X-100 with shaking at 180 rpm. Triton X-100 was required to prevent cell aggregation.

Detection of antibiotic activity.

The antibiotic activities of *Pseudomonas* strains were determined with *Bacillus subtilis* M168, *Pythium ultimum* MAFF-425494, or *F. oxysporum* MAFF103054 as the reporter. Cul-

tures of *Pseudomonas* strains were adjusted to OD₆₀₀ = 1.0 for the assay with *B. subtilis*, and 5-μl samples were spotted onto a plate with glycerol-casamino acid medium (Maurhofer et al. 1998) or NBglu. After overnight incubation at 28°C, cells of *Pseudomonas* strains were killed by UV irradiation on a transilluminator for 5 min. An overlay of *B. subtilis* revealed antibiotic production by growth inhibition zones. Cultures of *Pseudomonas* strains were adjusted to OD₆₀₀ = 1.5 for the assay with *P. ultimum* or *F. oxysporum*, 20-μl samples were streaked around the edge of the Davis-gly plate (for *F. oxysporum*) or an 1:1 mixture of Davis-gly and potato dextrose agar plate (for *P. ultimum*), and an inoculum of *P. ultimum* or *F. oxysporum* was transferred to the center of the plate. The plate was incubated at room temperature until *P. ultimum* or *F. oxysporum* reached the edge of the plate. As a quantitative measurement, DAPG was extracted with ethyl acetate from cell cultures (three replicates) grown in 50-ml flasks containing 10 ml of NBglu at 28°C for 20 h with shaking at 180 rpm. The extract was dried, dissolved in 100 μl of methanol, and analyzed by HPLC. DAPG production was quantified as described previously (Schnider-Keel et al. 2000).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Pseudomonas Genome database: www.pseudomonas.com
 Microbial Genome Annotation Pipeline database:
www.migap.org/index.php/en/aboutpipeline