Genome Sequence and Antifungal Activity of Two Niche-Sharing Pseudomonas protegens Related Strains Isolated from Hydroponics

Title

Genome sequence and antifungal activity of two niche-sharing *Pseudomonas protegens* related strains isolated from hydroponics.

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Abstract

This work reports the comparison of the genome sequence and the ability to inhibit fungal growth of two *Pseudomonas protegens* related strains that were isolated from the same hydroponic culture of lamb's lettuce. The two strains were very similar in their core genome but one strain, Pf4, contained three gene clusters for the production of secondary metabolites, *i.e.* pyoluteorin (*plt*), pyrrolnitrin (*prn*), and rhizoxin (*rzx*), that were missing in the other strain, Pf11. The difference between the two strains was not due to simple insertion events, but to a relatively complex differentiation focused on the accessory genomes. In dual culture assays, both strains inhibited nearly all tested fungal strains, yet Pf4 exerted a significantly stronger fungal growth inhibition than Pf11. In addition to the differences in the secondary metabolite production associated genes abundance, the genome of Pf4 was more stable, smaller in size and with a lower number of transposons. The preservation of a dynamic equilibrium within natural populations of different strains comprised in the same species but differing in their secondary metabolite repertoire and in their genome stability may be functional to the adaptation to environmental changes.

Keywords

biocontrol; floating system; population dynamic; pseudomonads; genome draft

Introduction

The rhizosphere environment hosts a complex of microorganisms that interact with plants in a multitude of ways [1]. Although vegetable crops are particularly sensitive to adverse interactions for various reasons associated with the intensity of cultivation [2], the impact of pathogens can be reduced, sometimes significantly, by manipulating characteristics of the rhizosphere, particularly its microbial community [3].

While not all root diseases can be avoided [4], their impact can be ~~decisively~~substantially limited by employing microorganisms as biological control agents (BCA) [5]. A particularly suited genus is *Pseudomonas* [6], intensely studied as a model for rhizosphere ecological studies and analysis of secondary metabolism [7], and for the ability to inhibit the growth of fungal and oomycete pathogens [8, 9].

Historically, the interest on pseudomonads as BCA descends from their dominance as root colonizer in disease suppressive soil [10], hence their use as BCA [11]. However, one of the common pitfall of single microbial inoculants concerns their survival in the environment and the consequent variability in the efficacy and frequent failure in the biocontrol [12], that triggered interest toward deciphering the dynamics of the microbial communities.

Microbial communities constitute a complex network of interactions, driven by the necessity of exploiting and controlling limited resources [13, 14]. These interactions can be competitive or mutualistic, and can take place ~~between~~amomg different species, different strains in the same species, or ~~between~~ members of the same species and strain [15]. Intraspecific diversity is relevant not only ~~for~~to driv~~e~~~~ing~~ the dynamic of the adaptation of the species over time to changing environmental conditions, but also for the peculiar aspects of cooperation and competition, that allow the preservation of apparently less fit strains. Very well known cases are those of bacteria that can take advantage of metabolites produced by other organisms and shift the cost of producing them, *e.g.* using heterologous siderophores [16]; or those of mutants in a quorum sensing regulator that become able to benefit from the protease activity of the enzymes secreted by their neighbours without the need to produce the enzymes themselves [17].

In addition to the emergence of mutants in a population, intraspecific diversity, as focused on this paper, may be associated with cohabitation of strains featuring very different secondary metabolite production. Co-existence of different strains with divergent metabolic features has been shown for example in mycorrhiza, concerning secretion of effector-like proteins and other symbiosis differentially expressed genes [18]; and in rumen, where the co-existence of multiple strains of *Ruminococcus albus* carrying distinct functional carbohydrate-active proteins was demonstrated to be important for efficient cellulose degradation [19].

The structural genomics aspects involved in intraspecific diversity in rhizosphere bacteria are still largely unknown, and we attempt to provide some data concerning *Pseudomonas protegens* ~~with~~in this paper. In a previous work, a group of *P. protegens* related strains were isolated from

hydroponic cultures of lamb's lettuce, ~~for~~according to their growth limiting activity against two strains of *Pythium aphanidermatum* and ~~a~~ two strains of *Rhizoctonia solani* [20]. One strain, Pf4, showed the strongest activity and was further selected for *in vivo* trials and analysis of the genome, that following to database searches resulted similar to that of the strains named Pf-5 and Os17, well known BCA isolated from other environments [21, 22] . With Pf4, however, other strains were isolated that according to marker gene analyses belonged to the same species of Pf4 but showed a more limited biological activity against *P. aphanidermatum* and *Rhizoctonia solani*. The aim of this work was to investigate the differences between one of those strains, named Pf11, and Pf4, to clarify the range of the biological activity against a larger number of fungal strains, determine the correlation with genomic features, and possibly obtain clues about their dynamics and significance in the intraspecific diversity of the natural populations of this important component of the microbial community of the rhizosphere.

Materials and methods

Pseudomonas strains and genomes

The *Pseudomonas* sp. Pf4 and Pf11 strains were isolated in 2009 from the roots of healthy *Valerianella locusta* (L.) Laterr. plants grown in a hydroponic farm in Friuli Venezia Giulia (FVG) region, north-eastern Italy. Genomic DNA was extracted from 1 ml of 24 hrs old cultures grown in Nutrient Broth with agitation using a Wizard DNA purification kit (Promega Italia, Padova, Italy) following the manufacturer's instructions. DNA was measured and checked for quality using a NanoDrop™ (NanoDrop products, Wilmington, DE, USA). Illumina™ libraries were prepared as described previously [23] and sent to the Istituto di Genomica Applicata (Udine, Italy) for sequencing on a Illumina™ Myseq™ with a 2×300 Reagent kit. Genomes were assembled using the A5-miseq pipeline [24] and annotated using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) and the RAST server [25]. The BioSample accessions are SAMN04554942 (for Pf4) and SAMN04554943 (for Pf11). A preliminary genome draft of Pf4 has been published [20], while no genomic information about Pf11 has been previously reported.

JspeciesWS [26] was used to measures the average nucleotide identity based on BLAST+ (ANIb) and MUMmer (ANIm), and correlation indexes of tetra-nucleotide signatures (Tetra), that indicate whether two genomes share genomic identities above or below the species embracing thresholds.

Orthologs and metabolic pathways

The NCBI-annotated sequences of Pf11 and Pf4 were compared for orthologs using the standalone version of the *Orthologous Matrix* tool (OMA; http://omabrowser.org/standalone/). The output was converted into a comparison table, using a custom Perl/Bash script.

Secondary metabolic pathways were investigated by verifying the presence of a selection of genes and gene clusters typical of *P. protegens* strains [27–29] in the NCBI annotation. The two genomes were also submitted to the *antiSMASH* 3.0 tool for rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences [30], for comparison with the hand-selected cluster findings. Structural and functional features of the two genomes were compared using Mauve [31], BUSCO [32], and some *ad hoc* Perl scripts.

Fungal growth inhibition tests

To test the ability of both *Pseudomonas* strains to inhibit fungal growth, a total of 15 fungal strains were assayed by the dual culture method according to [33]. The strains were freshly isolated and identified by rDNA sequencing as belonging to the following species: *Alternaria alternata, Aspergillus flavus, A. niger, Fusarium oxysporum* f. sp. *niveum, F. oxysporum* f. sp. *vasinfectum, F. verticillioides, Penicillium chrysogenum, P. griseofulvum, P. verrucosum, Ilyonectria europaea, I. robusta, Epicoccum nigrum, Neopestalotiopsis rosae, Phoma betae, Botritis cinerea, Colletotrichum* sp.

Nine repetitions of each fungal strain were placed in the centre of standard Petri dishes (internal diameter 85 mm) containing PDA supplemented with 3 g/l peptone and 2 g/l yeast extract, three of which were streaked at the sides, in a square shape, with Pf4, and another three with Pf11; the last three were the control samples, with the fungus alone. The Petri dishes were incubated at room temperature in a dim-lit environment. The dishes were photographed every 24 hours, for at least 10 days, and the diameter of each fungal colony was recorded (for the early square shapes, an average diameter was noted). In the statistical analysis, for each sample, the initial diameter of the inoculum was subtracted from the subsequent measures.

For each fungus run, a starting (*DPIini*) and ending (*DPIend*) time for the statistical tests were selected: *DPIini* was defined as the first day in which the diameters of all replicas were wider then 5 mm, while *DPIend* was defined as the first day in which at least one replica had reached the border of the Petri dish, the day in which most of the replicas of the control (fungus alone) culture stopped to grow, or the last sampling day.

One-way ANOVA and post hoc Tukey's test with 0.01% significance were performed for each observation between *DPIini* e *DPIend*. All statistical analysis was carried out using the "multcomp" package [34] of the R statistical computing language [35].

Results

Genome sequencing

The Illumina™ Sequencing of strain Pf11 DNA produced 3,727,137 reads, 300 nucleotides (nt) each, for a total of $1.1 \cdot 10^9$ nt, while sequencing of Pf4 DNA produced 1,914,469 reads, 300 nt each, for a total of $0.6 \cdot 10^9$ nt. The assembled Pf11 draft genome sequence resulted 7,053,517 nt

long in total, with a 62.0% G+C content, and consists of 125 contigs ranging from 605 to 1,372,031 nt in size (N50: 1,036,338), with a coverage of 205.3×. The assembled Pf4 draft genome sequence resulted 6,832,152 nt long in total, with a 62.5% G+C content, and consists of 36 contigs ranging from 605 to 1,018,138 nt in size (N50: 688,889), with a coverage of 100.9×.

The genome sequence draft of Pf11 contains 6154 predicted protein-coding sequences and 135 pseudogenes. In addition, 63 tRNA genes and 11 rRNA genes were identified. The genome sequence of Pf4 contains 5907 predicted protein-coding sequences and 61 pseudogenes. ~~In addition,~~ 62 tRNA genes and 11 rRNA genes ~~were identified~~.

Comparison of the Pf4 and Pf11 genomes.

Strains Pf4 and Pf11 are very similar to each other. Their 16SrRNA gene sequences are almost identical (1 nt difference in the entire sequence). Their pairwise ANIm, ANIb and Tetra value were 97.68, 97.08-97.31 and 0.99956, respectively, indicating that the strains are genomically related, well above the species embracing thresholds. The Pf4 and Pf11 genomes resulted also to share a large number of orthologous genes. The program OMA found 5534 orthologs, representing 89.9% of the Pf11 genome and 93.7% of the Pf4 genome. We selected the predicted protein sequences of 437 orthologs that are highly conserved among the gammaproteobacteria according to the BUSCO data-set [32], and estimated that the identical sequences were 261, while 100 had a single amino acid difference. In the total 153,633 amino acid residues resulting from concatenation of the 437 core protein sequences only 403 (0.26%) were different between Pf4 and Pf11. Differences among the two genomes were mostly found in the accessory genome; the OMA program listed 427 genes exclusive to Pf4 (Table 1 and S1) and 741 exclusive to Pf11 (Table 2 and S2).

According to a Tetra correlation searches of the JspeciesWS database [25], the genomes of Pf4 and Pf11 were closely related to that of Pf-5, a strain well known for its ability to suppresses plant diseases caused by soilborne plant pathogens [35 and references therein]. By using the complete sequence of Pf-5 as a reference (CP0000076.1; [22]), we carried out the scaffolding of 16 contigs of Pf4 (accounting for 6,802,786 nt, which correspond to >99.5% of the Pf4 estimated genome size) on one hand and, on the other, the scaffolding of 15 contigs of Pf11 (accounting for 6,934,975 nt, which correspond to >98.3% of the Pf11 estimated genome size). The sequence not included in the scaffolding (29,366 nts in 20 contigs for Pf4 and 118,542 nts in 110 contigs for Pf11) was found to predominantly encode transposases, phage related proteins, and other likely extrachromosomal elements, with no proteins related to the synthesis of secondary metabolites. These sequences were ~~as~~ not considered further and the following results concern the scaffolded contigs.

According to the genome alignment carried out with Mauve (shown in Figure 1), the two genomes have a strong colinearity and conservation. However, the alignment highlighted 96 sequence regions (larger than 1,000 nt) in the genome of Pf4 that were missing in Pf11 (460,862 nt total), and 68 in the genome of Pf11 that were missing in Pf4 (600,403 nt total). As shown ~~it can be~~

~~noticed~~ in Figure 1, in the Pf4 genome, region 4 in scaffold 1 and region 5 in scaffold 2, as well as the region of poor similarity and rearrangements located around and including region 3 in scaffold 4, contain many genes involved in secondary metabolism. Moreover, several strain specific polyketide synthase (PKS) could be located around and in region 1 in scaffold 8. Conversely, in the Pf11 genome, only the large region 1 in scaffold 1 was rich in genes involved in secondary metabolism.

Overall, the genome of Pf11 was about 200 kb (3%) larger than the genome of Pf4, the difference being related with a larger accessory genome. The count of genes annotated as conjugative protein and transposase sums 43 in Pf11 and only 4 in Pf4, suggesting that the presence of mobile elements is more extensive in Pf11.

Genes involved in the production of secondary metabolites

~~In a comparison for their potential in the production of secondary metabolites, t~~The two genomes ~~resulted~~were also similar for their potential in the production of secondary metabolites, yet with some significant differences. A summary of the comparison of the secondary metabolite gene content of the two strains is given in Table 3, and reported in full detail in Table S3.

The following n~~N~~ine gene clusters for antibiotic metabolites typical of *P. protegens* were found in both Pf4 and Pf11 strains, along with *gac*/*rsm* homologues and small regulatory RNAs: hydrogen cyanide (*hcn*), 2,4-diacetylphloroglucinol (*phl*), AprX protease (*apr*), pyoverdine (*pvd*), enantio-pyochelin (*pch*), hemophore biosynthesis (*has*), ferric-enterobactin receptor (*pfe*), orfamide A (*ofa*), and FitD toxin (*fit*). Conversely, t~~T~~hree clusters, *i.e.* pyoluteorin (*plt*), pyrrolnitrin (*prn*), and rhizoxin (*rzx*), were present only in Pf4.

Genes in the *hcn* cluster showed high similarity (between 91% and 98%) to those of *P. protegens* strain Pf-5 in the case of Pf11, while in the case of Pf4 the best matches were to those of strains *P.* sp. Os17 and St29 (95%–99%); similarly, the genes in the Pf11 *phl* cluster have high similarity (92%–98%) to those of *P. protegens* strain Pf-5 and to those of *P.* sp. Os17, and St29 and *P. protegens* strains in the case of Pf4 (90%–99%).

In both Pf-11 and Pf-4, high similarity to the PH1b, CMAA1215 and Os17 strains was found for the *apr* cluster (92–99%) and to Pf-5 for the cluster associated with the *gac*/*rsm* signal transduction (91–100%). The *pvd* cluster for pyoverdine, whose product has been reported in Pf-5 [36], is divided in four different loci, with varying levels of similarity; the largest locus spans genes A1395_30060–A1395_30155, with similarity ranging between 31% and 96%, in Pf11, and (NCBI ID) A1348_29340–A1348_29435, with similarity ranging between 35% and 100% in Pf4.

Clusters for enantio-pyochelin, fully conserved in Pf-5 [37], hemophore biosynthesis, ferric-enterobactin receptor and orfamide A were also found in both strains. The *fit* cluster [38], in the downstream region of the *rzx* cluster in Pf-5, has 88–97% identity in both cases to *P. protegens* Pf-5 homologous, and appears inverted compared to *P. protegens* strain Pf-5 and *P.* sp. Os17.

Differently from Pf4, the *plt* cluster for pyoluteorin and the *prn* cluster for pyrrolnitrin, typical antibiotic metabolites in *P. protegens*, as well as the *rzx* gene cluster encoding analogs of the antimitotic macrolide rhizoxin, are not present in Pf11.

In addition to the *P. protegens* typical metabolites, using the antiSMASH tool we found additional secondary metabolite production related genes in both Pf11 and Pf4, such as those involved in the synthesis of the antibiotic mitomycin, of the mixed ligand siderophore amychelin, and of the arylpolyene pigments (Table 4 and Table 5). In addition, antiSMASH found in Pf11 genome genes related to the synthesis of alginate that were not found in the Pf4~~11c~~ genome.

Fungal growth inhibition tests

Strains of all fungal species took at least 9 days to reach the plate border, therefore growth curves were constructed with data of 8 days of growth. The diameter of the fungal colonies grown for 9 days in the presence of strain Pf11 ranged from 22 mm (*P. verrucosum*) to 57 mm (*E. nigrum)*, while those grown in the presence of Pf4 ranged from 15 mm (*N. rosae*) to 53 mm (*A. niger*).

For all fungi except *A. niger* and *A. flavus*, a statistically significant inhibition effect was caused by both Pf4 and Pf11, after at least 2 to 4 days post inoculum (DPI) (Figure 2).

In a first group of fungi (*E. nigrum, Colletotrichum* sp.*, A. alternata, I. robusta, P. betae, P. verrucosum*), Pf4 inhibited fungal growth to a significantly larger extent than Pf11. Significant difference from the controls was determined for all these fungi since the 2nd DPI, while the difference between Pf4 and Pf11 was observed after the 2nd (*E. nigrum*), the 3rd (*Collectricum* sp., *A. alternata*), 5th (*I. robusta*) or 9th DPI (*P. verrucosum*).

For a second group of fungi (*F. verticillioides, F. oxysporum* f. sp. *vasinfectum, F. oxysporum* f. sp. *niveum, N. rosae, B.cinerea, P. chrysogenum and I.europaea*) Pf4 and Pf11 affected the growth of the fungi as compared to the controls, but there was no significant difference between Pf4 and Pf11.

In *A. niger,* the presence of Pf4 or Pf11 enhanced, rather than inhibit, the fungus growth rate, the difference to the controls becoming statistically different at the 7th DPI. No significant difference was observed between ~~the~~ Pf4 and Pf11.

Finally, in *A. flavus,* no statistical difference was found among Pf4, Pf11, and the controls.

Discussion

Intraspecific diversity is a relevant and still poorly understood factor in determining the dynamics and composition of the microbial communities [39]. The intraspecific diversity in secondary metabolite production of sympatric bacterial populations has a potential role in the dynamic equilibrium between resident microbiota, pathogens and BCAs and may affect the establishment of

durable biological control strategies. According to current view, secondary metabolites are a major component in the interface with the microbial community, as well as in the success of a wide range of biocontrol agents [14]. For *Pseudomonas protegens* and related strains, in particular, the production of secondary metabolites that interfere with fungal and bacterial pathogen growth is a prominent feature. The structure and biochemical target of many of those compounds, as well as the regulation of their production, have been deeply investigated. Less efforts have been committed to determine the range of target organism specificity of each compound and hence the effect on the non-plant-pathogenic microbial community of the diverse blend of secondary metabolites that is specifically produc~~vid~~ed by each strain.

In this work, we showed that the range of fungal organisms whose growth was inhibited by strains Pf4 and Pf11 is wide, as the growth of all fungal strains assayed in our work, with the notable exception of those belonging to the genus *Aspergillus*, was significantly reduced by the metabolites produced by both bacterial strains. Although this study does not provide information on the actual production of secondary metabolites, it reports ~~the evidence~~ that the biosynthesis genes for pyoluteorin, pyrrolnitrin, and rhizoxin (*rzx*) are encoded by the Pf4 genome only; their expression and synergistic effect with other metabolites would allow Pf4 to exert a growth inhibition on certain fungal strains stronger than Pf11. Conceivably, within the strain specific repertoire of diverse metabolites, some products have large antifungal range (i.e., HCN and DAPG), while others have a more specific target and synergistic effect. The relevance of such a synergistic~~synergstic~~ effect is in agreement with the report of Takeuchi and coworkers [21], that rhizoxin analogs contribute to the biocontrol activity of *P. protegens* strain Os17 as compared with strain St29, where the relevant gene cluster was missing.

According to the results of the genomic analysis presented here, the larger metabolite repertoire of Pf4 is associated with a smaller accessory genome and a lower number of transposons. Mobile DNA elements can generate significant variability in bacteria and contribute to improve their fitness, promoting incorporation of foreign DNA and rearrangements in the host chromosome that may affect the phenotype. However transposon proliferation introduce deleterious mutations and the incorporation of mobile DNA may produce an excessive growth of the genome, that is therefore limited by recombination and selection [40]. The genomes of the two strains therefore differ not only in their content of biosynthetetic gene clusters, but also in structural features that are related with their evolution and adaptation.

The isolation from the same environment of strains that are taxonomically strictly related, yet significantly different in their interaction with other microorganisms, suggested that seemingly less fit bacterial strains, with narrower metabolite production patterns, have an opportunity to survive along with more competitive ones. It is tempting to speculate that while the strong fungal growth inhibitory activity of Pf4 on one hand advocates a role of deployment against fungal pathogens, on the other it has the potential for altering the microorganism community; that might associate with

an eventual decline of the Pf4 population itself due to the need to adapt to changing conditions. Under this view, a bacterium like Pf11, producing a more limited array of metabolites, might have a lower impact on the composition of the microbial community. In other words, the survival of the species could benefit from a dynamism of the prevalence of Pf4- and Pf11-like strains within the community.

The results of this work highlight the contrast between a classification based on taxonomic markers and one based on ecological roles; species that may appear homogeneous on a taxonomical level might on the contrary ~~present~~show a high ~~level~~degree of heterogeneity in terms of interactions with other microorganisms. A dynamic equilibrium among different strains comprised in the same species, *i.e.* those that allow the maximum exploitation of competitive features based on secondary metabolites and those that preserve a more complex microbial community, may be functional to the evolutionary success of the species.

An effective analysis of microbial diversity in ecological complex system needs to take into account the concepts outlined above. Although barcoding using taxonomically informative genes such as ribosomal DNA is presently the most widely used approach to characterize complex microbial communities, it severely underestimates the diversity of the communities. Indeed, it would be interesting to know to what extent bacteria that are indistinguishable using rDNA and other gene markers, but differ significantly in the genetic features that control interaction with other microorganisms, can coexist in the same environment. When referring to *P. protegens*, a species comprising strains that have a significant production of bio-active secondary metabolites, the intra-species diversity and variability may play a major role in determining the composition of the microbial community.

The cohabitation of different strains that are strictly taxonomically related and share a prevalent fraction of their genomes, yet with significantly different secondary metabolite profiles, is functional to their continued coevolution. A continuous trade of horizontally transferred genetic material needs to be fuelled with new genetic information and, to this end, the intra-species diversity plays an instrumental role. According to the results presented in this paper, the putative production of pyoluteorin, pyrrolnitrin, and rhizoxin, typical antibiotic metabolites in *P. protegens,* as supported by the presence of biosynthsys genes in the genome, is relevant in determining the fungal growth inhibition pattern of competitive *P. protegens* strains. Clusters *prn, rzx* and *plt* were found only in Pf4 scaffolds 8 and 4, along with two regions on scaffolds 1 and 5 coding for secondary metabolites; therefore the difference between the two strains can't be attributed to a simple insertion event, but implies a relatively complex differentiation focused on the accessory genomes. In contrast to the core genome, the evolution in the accessory genomes progresses exploiting primarily horizontal gene transfer consequent to multiple invasion of foreign DNA, that could be more or less stably integrated in the genome. *P. protegens* strains have the largest genomes among the bacteria of the fluorescens group and in general among *Pseudomonas* (whose

genomes range from 4.17 to 8.6 Mb). Conceivably, larger genomes allow to accommodate metabolic gene clusters conferring environmental fitness advantages that compensate for the relax of an otherwise strict genome size constrains. In particular, with 7.05 Mb, Pf11 stands at the high range of the genomes size allowed for the species. It can be speculated that genome expansion (with horizontal acquisition of genes) and contraction is a dynamic process that lead to a more stable genome. In this view, the Pf11 genome, with its larger size and the larger number of transposable elements appears to be in more dynamic evolutionary stage as compared to Pf4 genome that already gained a richer pattern of secondary metabolite production associated gene clusters.

References

1. Raaijmakers JM, Paulitz TC, Steinberg C, et al (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil 321:341–361. doi: 10.1007/s11104-008-9568-6
2. Colla P, Gilardi G, Gullino ML (2012) A review and critical analysis of the European situation of soilborne disease management in the vegetable sector. Phytoparasitica 40:515–523. doi: 10.1007/s12600-012-0252-2
3. Mazzola M (2002) Mechanisms of natural soil suppressiveness to soilborne diseases. Antonie Van Leeuwenhoek 81:557–564. doi: 10.1023/A:1020557523557
4. Vallance J, Déniel F, Le Floch G, et al (2011) Pathogenic and beneficial microorganisms in soilless culture. Agron Sustain Dev 31:191–203
5. Handelsman J, Stabb E V (1996) Biocontrol of soilborne plant pathogens. Plant Cell 8:1855–1869
6. Weller DM (2007) Pseudomonas biocontrol agents of soilborne pathogens: Looking back over 30 years. Phytopathology 97:250–256. doi: 10.1094/PHYTO-97-2-0250
7. Couillerot O, Prigent-Combaret C, Caballero-Mellado J, Moënne-Loccoz Y (2009) *Pseudomonas fluorescens* and closely-related fluorescent pseudomonads as biocontrol agents of soil-borne phytopathogens. Lett Appl Microbiol 48:505–512. doi: 10.1111/j.1472-765X.2009.02566.x
8. Rankin L (1994) Evaluation of rhizosphere bacteria for biological control of *Pythium* root rot of greenhouse cucumbers in hydroponic culture. Plant Dis 78:447. doi: 10.1094/PD-78-0447
9. Van Os EA (1999) Closed soilless growing systems: A sustainable solution for Dutch greenhouse horticulture. Water Sci Technol 39:105–112. doi: 10.1016/S0273-1223(99)00091-8
10. Schroth MN, Hancock JG (1982) Disease-suppressive soil and root-colonizing bacteria. Science 216:1376–1381. doi: 10.1126/science.216.4553.1376

11. Gómez Expósito R, de Bruijn I, Postma J, Raaijmakers JM (2017) Current insights into the role of rhizosphere bacteria in disease suppressive soils. Front Microbiol 8:2529. doi: 10.3389/fmicb.2017.02529

12. Lugtenberg B, Leveau J (2007) Biocontrol of plant pathogens: principles, promises, and pitfalls. In: Pinton R, Varanini Z, Nannipieri P (eds) The rhizosphere: biochemistry and organic substances at the soil–plant interface. CRC Press, Boca Raton, pp 267–296.

13. Hibbing ME, Fuqua C, Parsek MR, Peterson SB (2010) Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Microbiol 8:15–25. doi: 10.1038/nrmicro2259

14. Stubbendieck RM, Straight PD (2016) Multifaceted Interfaces of bacterial competition. J Bacteriol 198:2145–2155. doi: 10.1128/JB.00275-16

15. Hibbing ME, Fuqua C, Parsek MR, Peterson SB (2010) Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Microbiol 8:15–25. doi: 10.1038/nrmicro2259

16. Khan A, Geetha R, Akolkar A, et al (2006) Differential cross-utilization of heterologous siderophores by nodule bacteria of *Cajanus cajan* and its possible role in growth under iron-limited conditions. Appl Soil Ecol 34:19–26. doi: 10.1016/j.apsoil.2005.12.001

17. Sandoz KM, Mitzimberg SM, Schuster M (2007) Social cheating in *Pseudomonas aeruginosa* quorum sensing. Proc Natl Acad Sci 104:15876–15881. doi: 10.1073/pnas.0705653104

18. Chen ECH, Morin E, Beaudet D, et al (2018) High intraspecific genome diversity in the model arbuscular mycorrhizal symbiont *Rhizophagus irregularis*. New Phytol. doi: 10.1111/nph.14989

19. Grinberg IR, Yin G, Borovok I, et al (2015) Functional phylotyping approach for assessing intraspecific diversity of *Ruminococcus albus* within the rumen microbiome. FEMS Microbiol Lett 362:1–10. doi: 10.1093/femsle/fnu047

20. Moruzzi S, Firrao G, Polano C, et al (2017) Genomic-assisted characterisation of *Pseudomonas* sp. strain Pf4, a potential biocontrol agent in hydroponics. Biocontrol Sci Technol 27:969–991. doi: 10.1080/09583157.2017.1368454

21. Takeuchi K, Noda N, Katayose Y, et al (2015) Rhizoxin analogs contribute to the biocontrol activity of a newly isolated *Pseudomonas* strain. Mol Plant-Microbe Interact 28:333–342. doi: 10.1094/MPMI-09-14-0294-FI

22. Paulsen IT, Press CM, Ravel J, et al (2005) Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. Nat Biotechnol 23:873–878. doi: 10.1038/nbt1110

23. Scortichini M, Marcelletti S, Ferrante P, Firrao G (2013) A Genomic redefinition of *Pseudomonas avellanae* species. PLoS One 8:e75794. doi: 10.1371/journal.pone.0075794

24. Tritt A, Eisen JA, Facciotti MT, Darling AE (2012) An integrated pipeline for de novo assembly of microbial genomes. PLoS One 7:e42304. doi: 10.1371/journal.pone.0042304

25. Aziz RK, Bartels D, Best AA, et al (2008) The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics 9:75. doi: 10.1186/1471-2164-9-75

26. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J (2016) JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics 32:929–931. doi: 10.1093/bioinformatics/btv681

27. Blankenfeldt W, Parsons JF (2014) The structural biology of phenazine biosynthesis. Curr Opin Struct Biol 29:26–33. doi: 10.1016/j.sbi.2014.08.013

28. Loper JE, Hassan KA, Mavrodi D V., et al (2012) Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. PLoS Genet 8:e1002784. doi: 10.1371/journal.pgen.1002784

29. Mavrodi D V., Peever TL, Mavrodi O V., et al (2010) Diversity and evolution of the phenazine biosynthesis pathway. Appl Environ Microbiol 76:866–879. doi: 10.1128/AEM.02009-09

30. Weber T, Blin K, Duddela S, et al (2015) AntiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43:W237–W243. doi: 10.1093/nar/gkv437

31. Darling ACE, Mau B, Blattner FR, Perna NT (2004) Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res 14:1394–1403. doi: 10.1101/gr.2289704

32. Simão FA, Waterhouse RM, Ioannidis P, et al (2015) BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31:3210–2. doi: 10.1093/bioinformatics/btv351

33. Gravel V, Martinez C, Antoun H, Tweddell RJ (2005) Antagonist microorganisms with the ability to control *Pythium* damping-off of tomato seeds in rockwool. Biocontrol 50:771–786. doi: 10.1007/s10526-005-1312-z

34. Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biometrical J 50:346–363. doi: 10.1002/bimj.200810425

35. R Development Core Team (2007) R: a Language and Environment for Statistical Computing. R Found. Stat. Comput. Vienna (2007).

36. Gross H, Loper JE (2009) Genomics of secondary metabolite production by *Pseudomonas* spp. Nat Prod Rep 26:1408. doi: 10.1039/b817075b

37. Youard ZA, Mislin GLA, Majcherczyk PA, et al (2007) *Pseudomonas fluorescens* CHA0 produces enantio-pyochelin, the optical antipode of the *Pseudomonas aeruginosa* siderophore pyochelin. J Biol Chem 282:35546–35553. doi: 10.1074/jbc.M707039200

38. Péchy-Tarr M, Bruck DJ, Maurhofer M, et al (2008) Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. Environ Microbiol 10:2368–2386. doi: 10.1111/j.1462-2920.2008.01662.x

39. Xavier JB, Foster KR (2007) Cooperation and conflict in microbial biofilms. Proc Natl Acad Sci U S A 104:876–81. doi: 10.1073/pnas.0607651104

40. Dolgin ES, Charlesworth B (2006) The fate of transposable elements in asexual populations. Genetics 174:817–827. doi: 10.1534/genetics.106.060434

Figure and table legends

Figure 1 – Mauve alignment of Pf11 and Pf4. The red dots mark unique regions larger than 10,000 nt.

Figure 2 – Results of the inhibition assays. The bars represent the diameters means for Control, Pf11, Pf4 replicas observed at the last date relevant for statistical analysis (*DPIend*). Error bars are the standard deviation, while different letters indicate different means based on post hoc Tukey test at 0.01 level of significance. The number at the top right of each graph specify the *DPIend*.

Table 1 – Highlights of OMA-isolated genes exclusive to Pf4. An exhaustive list is present in the supplemental data, Table S1.

Table 2 – Highlights of OMA-isolated genes exclusive to Pf11. An exhaustive list is present in the supplemental data, Table S2.

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Table 3 – Summary of the sequence analysis of gene clusters for the synthesis of antibiotics, exoenzyme, cyclic lipopeptide, siderophores, and toxin, and of Gac/Rsm homologues in *P. protegens* related strains Pf11 and Pf4 and similarities to those in *P. protegens* strains (Pf-5, PH1b) and other closely related *Pseudomonas* sp. (CMAA1215, NFPP17, Os17). A more detailed list of genes is present in Table S3.

Table 4 – Gene clusters in Pf11 determined by antiSMASH 3.0 web tool. Under the “Most similar known cluster” column, the percentage is the proportion of genes that show similarity.

Table 5 – Gene clusters in Pf4 determined by antiSMASH 3.0 web tool. Under the “Most similar known cluster” column, the percentage is the proportion of genes that show similarity.

Supplementary Materials

Figure S1 – Colony diameters (*y*-axis) for each replica and for each observation day (*x*-axis).

Table S1 – Genes exclusive to Pf4 as determined by the stand-alone OMA program.

Table S2 – Genes exclusive to Pf11 as determined by the stand-alone OMA program.

Table S3 – Sequence analysis of gene clusters for the synthesis of antibiotics, exoenzyme, cyclic lipopeptide, siderophores, and toxin, and of Gac/Rsm homologues in *P. protegens* Pf11 and Pf-4 and similarities to those in *P. protegens* strains (Pf-5, PH1b) and other closely related *Pseudomonas* sp. (CMAA1215, NFPP17, Os17).

Gene clusters present only in Pf-4 are: pyoluteorin (*plt*), pyrrolnitrin (*prn*), rhizoxin (*rzx*). Gene clusters present in both are: hydrogen cyanide (*hcn*), 2,4-diacetylphloroglucinol (*phl*), AprX protease (*apr*), *gac*/*rsm* homologues, small regulatory RNAs, pyoverdine (*pvd*), enantio-pyochelin (*pch*), hemophore biosynthesis (*has*), ferric-enterobactin receptor (*pfe*), orfamide A (*ofa*), and FitD toxin (*fit*).

Figure 1

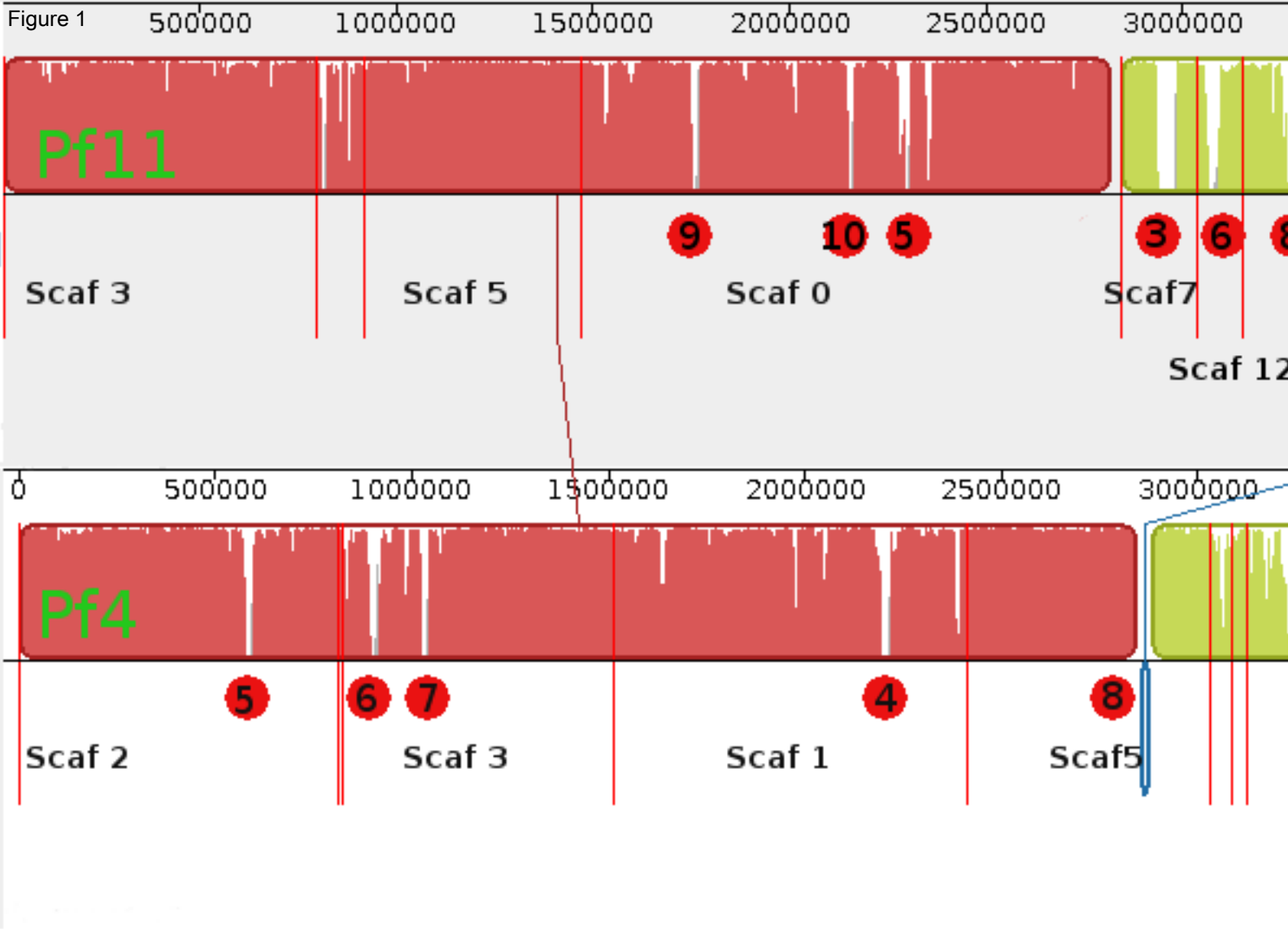
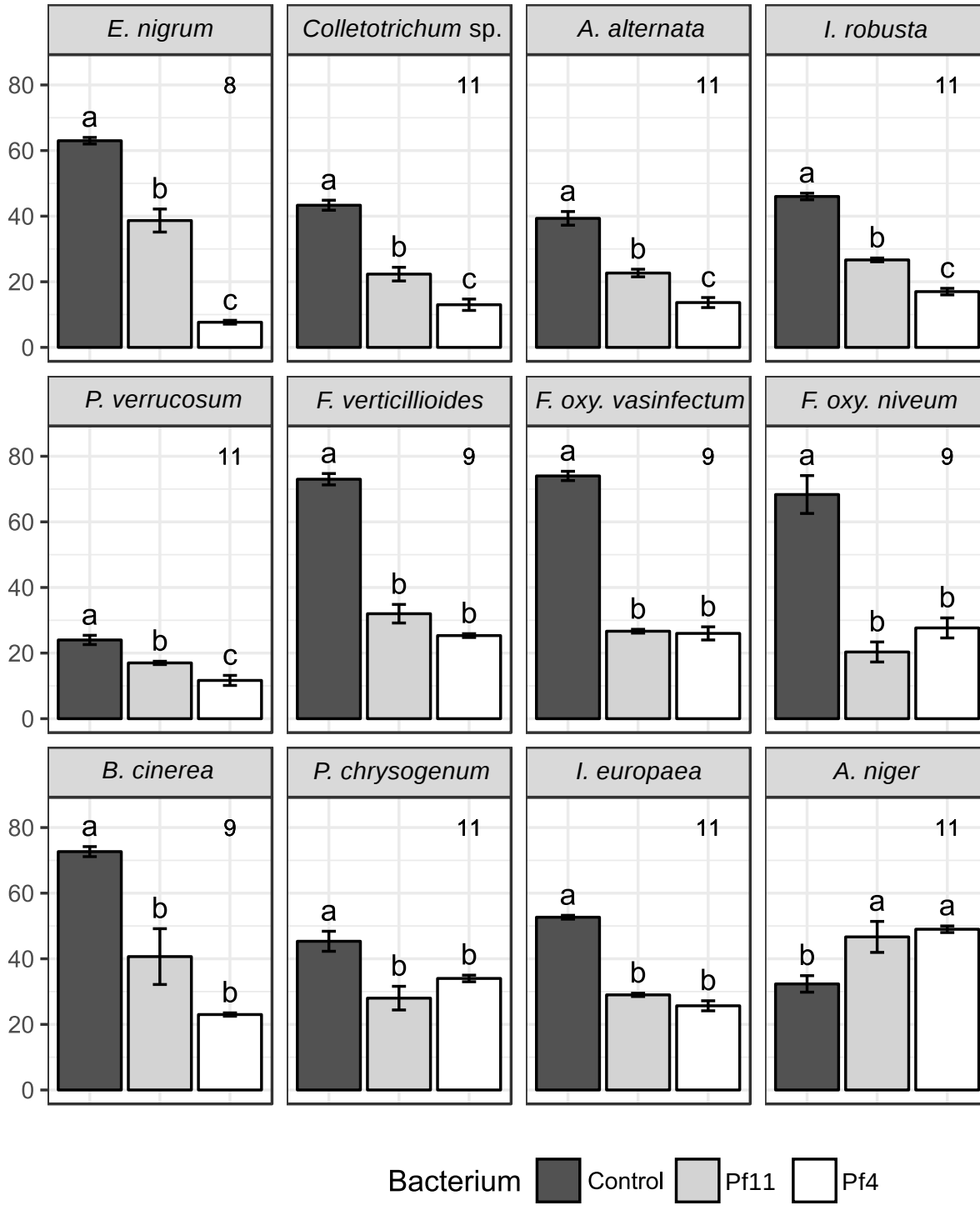

500000
1000000
1500000
2000000
2500000
3000000
Pf11
9
10
5
3
6
Scaf 3
Scaf 5
Scaf 0
Scaf7
Scaf 12
0
500000
1000000
1500000
2000000
2500000
3000000
Pf4
5
6
7
4
8
Scaf 2
Scaf 3
Scaf 1
Scaf5

Figure2


E. nigrum
8
Colletotrichum sp.
11
A. alternata
11
I. robusta
11
P. verrucosum
11
F. verticillioides
9
F. oxy. vasinfectum
9
F. oxy. niveum
9
B. cinerea
9
P. chrysogenum
11
I. europaea
11
A. niger
11
0
20
40
60
80
a
b
c
Bacterium
Control
Pf11
Pf4

Supplementary Figure 1

Supplementary Table 1

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Supplementary Material

tableS1-pf4-exclusive.csv

Supplementary Table 2

Supplementary Table 3

Table1

Gene code	Description
A1348_00295	iron dicitrate transport regulator FecR
A1348_01100, A1348_12715	antitoxin
A1348_01105	plasmid maintenance protein
A1348_05325	immunity protein
A1348_06835	organic radical-activating protein
A1348_07960	nitrilotriacetate monooxygenase
A1348_08840	RTX toxin
A1348_11565	ferredoxin
A1348_12720	addiction module toxin RelE
A1348_12950	pesticin immunity protein
A1348_15975	p-hydroxycinnamoyl CoA hydratase/lyase
A1348_16040	Vanillate O-demethylase oxidoreductase
A1348_16045	Rieske (2Fe-2S) protein
A1348_16275	nucleoid-associated protein YejK
A1348_17340	antibiotic ABC transporter permease
A1348_21675	nitric oxide synthase
A1348_22195	agmatine deiminase
A1348_25120	Holliday junction resolvase
A1348_25380	plasmid stabilization protein ParE
A1348_27055	flavin reductase
A1348_27075	monodechloroaminopyrrolnitrin synthase PrnB
A1348_29440	chemotaxis protein
A1348_30105, A1348_12615	large adhesive protein

Table2

Gene code	Description
A1395_07930	methanobactin biosynthesis cassette protein MbnB
A1395_08725	acriflavin resistance protein
A1395_08815, A1395_21000	DNA repair protein RadC
A1395_09195	multidrug transporter
A1395_09200	multidrug efflux RND transporter permease subunit
A1395_16665	phenol degradation protein meta
A1395_17490, A1395_29460	addiction module toxin RelE
A1395_17495	toxin-antitoxin system protein
A1395_17640	cytotoxic translational repressor of toxin-antitoxin stability system
A1395_20785	coproporphyrinogen III oxidase
A1395_21095	nitrilase
A1395_22460	metal-chelation protein CHAD
A1395_25370	prevent-host-death protein
A1395_27990	host specificity protein
A1395_28685, A1395_28745	lysozyme
A1395_29380	large adhesive protein
A1395_29465	antitoxin of toxin-antitoxin stability system
A1395_31465	spermidine/putrescine ABC transporter substrate-binding protein
A1395_31485	nitrate reductase
A1395_31570	SfnB family sulfur acquisition oxidoreductase
nusA	transcription termination/antitermination protein NusA

Table3

Pf-11 NCBI Gene ID (A1395_)	Pf-4 NCBI Gene ID (A1348_)	Gene name (Pf5 equiv. PFL ID)	Pf-11 Locus	Pf-4 Locus
hcn gene cluster (for hydrogen cyanide) – present in both				
10425–10415	23065–23075	*hcnA* (2577)–*hcnC* (2579)	1: 991726–994695 (–)	6: 391003–393972 (+)
plt gene cluster (for pyoluteorin) – only in Pf-4				
–	17270–17350	*pltM* (2784)–*pltP* (2800)	–	4: 360091–390616
prn gene cluster (for pyrrolnitrin) – only in Pf-4				
–	27080–27065	*prnA* (3604)–*prnD* (3607)	–	8: 326813–332375
phl gene cluster (for 2,4-diacetylphloroglucinol) – present in both				
18635–18670	10485–10520	*phlH* (5951)–*phlE* (5958)	3: 364619–372851	2: 363678–371910
apr gene cluster (for AprX protease) – present in both				
08470–08450	26990–26970	*aprA* (3210)–*aprF* (3206)	1: 533253–539877 (–)	8: 303649–310279 (–)
Gac/Rsm homologues – present in both				
13645	03275	*gacS* (4451)	2: 326117–328870 (+)	0: 690217–692970 (–)
21170	25980	*gacA* (3563)	4: 104938–105522 (–)	7: 486282–486866 (+)
13900	03020	*rsmA* (4504)	2: 377278–377466 (–)	0: 641626–641814 (+)
17930	09780	*rsmE* (2095)	3: 220271–220990 (+)	2: 219078–219797 (+)
24025	15270	*retS* (0664)	5: 78482–81268 (+)	3: 607391–610177 (–)
26950	28385	*ladS* (5426)	6: 187267–189633 (–)	9: 172345–174711 (+)
Small regulatory RNAs – present in both				
N.A.	N.A.	*rsmZ* (6285)	0: 514076–513951 (–)	1: 506535–506661 (+)
N.A.	N.A.	*rsmY* (6291)	3: 74313–74197 (–)	2: 73788–73906 (+)
N.A.	N.A.	*rsmX* (6289)	10: 33390–33506 (+)	10:86797–86915 (+)
pvd gene cluster (for pyoverdine) – present in both				
07080–07085	17855–17860	*pvdQ* (2902)–*fpvR* (2903)	1: 189376–192763	4: 506592–509979
30155–30060	29340–29435	*pvdA* (4079)–PFL_4097	10: 46820–92493	10: 26184–71830

12240–12290	04660–04610	PFL_4169–*pvdH* (4179)	2: 17612–26974	10: 56263–59334 (–) 0: 990920–999310
12360–12370	04555–04545	*pvdL* (4189)–*pvdY* (4191)	2: 41461–55794	0: 962639–976972
pch cluster (for enantio-pyochelin) – present in both				
30475–30520	15840–15885	*pchR* (3497)–*pchA* (3488)	11: 53981–72965	4: 49492–68476
has gene cluster (for hemophore biosynthesis) – present in both				
26720–26690	28615–28645	*hasI* (5380)–*hasF* (5374)	6: 128190–138010 (–)	9: 223960–233779 (+)
pfe gene cluster (for ferric-enterobactin receptor) – present in both				
10085–10095	23430–23420	*pfeR* (2665)–*pfeA* (2663)	1: 916810–921183 (+)	6: 470135–474508 (–)
ofa gene cluster (for orfamide A) – present in both				
27845–27835	18430–18420	*ofaA* (2145)–*ofaC* (2147)	7: 7700–42217 (–)	5: 7709–42188 (–)
fit gene cluster (for FitD toxin) – present in both				
08015–07980	26560–26525	*fitA* (2980)–*fitH* (2987)	1: 402656–424286	8: 180030–201661
rzx gene cluster (for rhizoxin) – only in Pf-4				
–	26520–26475	PFL_2988–*rzxA* (2997)	–	8: 99945–179906

Table4

Cluster	Type	From	To	Most similar known cluster	MIBiG BGC-ID
scaffold 0					
Cluster 1	T1pks	269602	315724	Alginate biosynthetic g.c.* (100%)	BGC0000726 c1
Cluster 2	Bacteriocin	604419	615309	-	-
Cluster 3	Bacteriocin	643914	655860	-	-
scaffold 1					
Cluster 4	Thiopeptide-Lantipeptide-Bacteriocin	60239	100470	-	-
Cluster 5	Bacteriocin	387369	398181	-	-
Cluster 6	Nrps	1101588	1153056	Mitomycin biosynthetic g.c.* (5%)	BGC0000915 c1
scaffold 10					
Cluster 7	Nrps	28880	92959	Pyoverdine biosynthetic g.c.* (31%)	BGC0000413 c1
scaffold 11					
Cluster 8	Nrps	35259	89304	Amychelin biosynthetic g.c.* (12%)	BGC0000300 c1
scaffold 2					
Cluster 9	Nrps	21461	74477	Pyoverdine biosynthetic g.c.* (16%)	BGC0000413 c1
Cluster 10	Nrps	503749	554778	-	-
scaffold 3					
Cluster 11	T3pks	350414	391463	2,4-Diacetylphloroglucinol biosynthetic g.c.* (87%)	BGC0000281 c1
Cluster 12	Bacteriocin	629699	640544	-	-
scaffold 5					
Cluster 13	Arylpolyene	294021	337638	APE Vf biosynthetic g.c.* (40%)	BGC0000837 c1
scaffold 7					
Cluster 14	Nrps	1	62217	Orfamide biosynthetic g.c.* (70%)	BGC0000399 c1

*g.c.: “gene cluster”.

Table5

Cluster	Type	From	To	Most similar known cluster	MIBiG BGC-ID
scaffold 0					
Cluster 1	Nrps	464199	515228	-	-
Cluster 2	Nrps	943956	996972	Pyoverdine biosynthetic g.c.* (17%)	BGC0000413 c1
scaffold 1					
Cluster 3	Bacteriocin	597423	608313	-	-
Cluster 4	Bacteriocin	636903	648849	-	-
scaffold 10					
Cluster 5	Nrps	25701	89768	Pyoverdine biosynthetic g.c.* (27%)	BGC0000413 c1
scaffold 2					
Cluster 6	T3pks	349473	390522	2,4-Diacetylphloroglucinol biosynthetic g.c.* (87%)	BGC0000281 c1
Cluster 7	Bacteriocin	644287	655132	-	-
scaffold 3					
Cluster 8	Arylpolyene	350533	394150	APE Vf biosynthetic g.c.* (40%)	BGC0000837 c1
scaffold 4					
Cluster 9	Nrps	30770	84815	Amychelin biosynthetic g.c.* (12%)	BGC0000300 c1
Cluster 10	T1pks	344776	397525	Pyoluteorin biosynthetic g.c.* (100%)	BGC0000127 c1
Cluster 11	Lantipeptide-Bacteriocin	396010	420165	-	-
scaffold 5					
Cluster 12	Nrps	1	62188	Orfamide biosynthetic g.c.* (70%)	BGC0000399 c1
scaffold 6					
Cluster 13	Nrps	230297	281765	Mitomycin biosynthetic g.c.* (3%)	BGC0000915 c1
scaffold 8					
Cluster 14	Transatpks	79945	198849	Rhizoxins biosynthetic g.c.* (12%)	BGC0001112 c1
Cluster 15	Other	309674	350759	Pyrrolnitrin biosynthetic g.c.* (100%)	BGC0000924 c1

*g.c.: “gene cluster”.