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The Genomic Sequence of *Pseudomonas fluorescens* Pf-5: Insights Into Biological Control

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ABSTRACT

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The complete sequence of the 7.07 Mb genome of the biological control agent *Pseudomonas fluorescens* Pf-5 is now available, providing a new opportunity to advance knowledge of biological control through genomics. *P. fluorescens* Pf-5 is a rhizosphere bacterium that suppresses seedling emergence diseases and produces a spectrum of antibiotics toxic to plant-pathogenic fungi and oomycetes. In addition to six known secondary metabolites produced by Pf-5, three novel secondary metabolite biosynthesis gene clusters identified in the genome could also contribute to biological control. The genomic sequence provides numerous

clues as to mechanisms used by the bacterium to survive in the spermosphere and rhizosphere. These features include broad catabolic and transport capabilities for utilizing seed and root exudates, an expanded collection of efflux systems for defense against environmental stress and microbial competition, and the presence of 45 outer membrane receptors that should allow for the uptake of iron from a wide array of siderophores produced by soil microorganisms. As expected for a bacterium with a large genome that lives in a rapidly changing environment, Pf-5 has an extensive collection of regulatory genes, only some of which have been characterized for their roles in regulation of secondary metabolite production or biological control. Consistent with its commensal lifestyle, Pf-5 appears to lack a number of virulence and pathogenicity factors found in plant pathogens.

Recently, the genomic sequence of the biological control agent *Pseudomonas fluorescens* Pf-5 was completed (47). The genomic sequence of Pf-5 is the first published for a biological control agent for plant disease. In this symposium paper, we briefly review the literature describing Pf-5, describe aspects of the genomic sequence that are particularly relevant to the capacity of Pf-5 to suppress plant disease, and provide our perspectives on how the genomic sequence data are advancing knowledge of biological control.

Plant disease suppression by *P. fluorescens* Pf-5. Pf-5 was isolated from the cotton rhizosphere and first described for its capacity to suppress seedling diseases of cotton caused by *Rhizoctonia solani* (17) and *Pythium ultimum* (18). *R. solani* and *Pythium ultimum* are widespread pathogens with broad host ranges that constrain food and fiber production worldwide (34). The pathogens are the major causes of seed rot and seedling death of cotton and many other crop plants. Since Pf-5 was first described, it has been shown to suppress these pathogens on a variety of plant hosts including cucumber, pea, and maize (26; M. D. Henkels and J. E. Loper, unpublished data). Furthermore, Pf-5 suppresses a number of other soilborne or residue-borne fungal pathogens. For example, when inoculated onto wheat straw residue, Pf-5 suppresses ascocarp formation by the tan spot pathogen of wheat, *Pyrenophora tritici-repentis*, thereby decreasing inoculum available for infection of the subsequent wheat crop

(49). On turfgrass, Pf-5 suppresses dollar spot caused by *Sclerotinia homoeocarpa* and leaf spot caused by *Drechslera poae*, which are destructive and widespread diseases affecting golf courses, home lawns, and amenity turf areas (56). Pf-5 also suppresses Fusarium crown and root rot of tomato, caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (61), and seed piece decay of potato caused by the bacterial pathogen *Erwinia carotovora* (70). Because of the wide variety of diseases it suppresses, Pf-5 is well recognized by biological control researchers around the world, and commonly is included as a reference strain in studies of biocontrol agents (21,25,38,61,70).

Secondary metabolite production by Pf-5. Pf-5 produces at least seven secondary metabolites, including pyrrolnitrin (17), pyoluteorin (18), 2,4-diacetylphloroglucinol (44), hydrogen cyanide (26), the siderophores pyochelin (or related compounds) and pyoverdine, and a lipopeptide (H. Gross, J. E. Loper, and W. Gerwick, unpublished data). In addition, two putative metabolites are assumed to exist due to the presence of biosynthetic gene clusters in the Pf-5 genome (47). Five of these secondary metabolites are known to be produced by *P. fluorescens* strain CHA0, a rhizosphere bacterium isolated in Switzerland whose biological control characteristics, mechanisms of disease suppression, and regulatory networks controlling secondary metabolite production have been extensively characterized (14). Some other biological control strains produce a subset of metabolites produced by Pf-5, and their broad spectrum antibiotic activities have been reviewed elsewhere (52).

Regulation of secondary metabolite production. Production of secondary metabolites by strains CHA0 or Pf-5 is controlled by complex regulatory networks that respond to environmental and density-dependent signals and are coupled to the physiological status of the bacterium. Loci that regulate the production of anti-

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fungal metabolites in the strains include a two-component regulatory system encoded by *gacS* and *gacA* (9,30); the RNA-binding proteins RsmA and RsmE (4,54); the regulatory RNAs *rsmX* (20), *rsmY* (65), and *rsmZ* (15); the sigma factors σ^s (58) and σ^{54} (48); the cofactor PQQ (59); the anaerobic regulator *anr* (29); the translation initiation factor IF3 (3); the protease Lon (69); and *ptsP*, a paralog of sugar phosphotransferase enzyme I (67). Many of these loci control multiple phenotypes including stress response in *P. fluorescens* (58,63,68,69), indicating that regulation of antibiotic production is intricately enmeshed in the physiology of the bacterial cell. Secondary metabolite production does not appear to be controlled by a classic quorum-sensing system based upon *N*-acyl homoserine lactones in these strains, but at least two of the antibiotics—pyoluteorin and 2,4-diacetylphloroglucinol—serve as autoinducers of their own production (1,5,60).

How is Pf-5 related to other biological control strains of *P. fluorescens*? Biological control strains of *P. fluorescens* have

been isolated from soils and plant surfaces all over the world, and they comprise a diverse group of bacteria. Nevertheless, biological control strains fall into distinct groups defined by biochemical and molecular criteria. Studies evaluating collections of biocontrol strains using these criteria consistently place Pf-5 in a group with CHA0 and other pyoluteorin and 2,4-diacetylphloroglucinol-producing strains (37 and references therein).

The genome of Pf-5. At 7.07 Mb, the genome of Pf-5 is the largest *Pseudomonas* genome sequenced to date (7,13,19,42,64). It is composed of a single circular chromosome with 6,144 predicted genes (Fig. 1). Below, we provide a brief overview of the general features of the Pf-5 genome and highlight aspects of the genome of particular relevance to environmental fitness and biological control. The Pf-5 genomic sequence data discussed below have been published previously (47).

Environmental fitness. Pf-5 has the capacity to colonize seed and root surfaces, processes that involve the acquisition of diverse

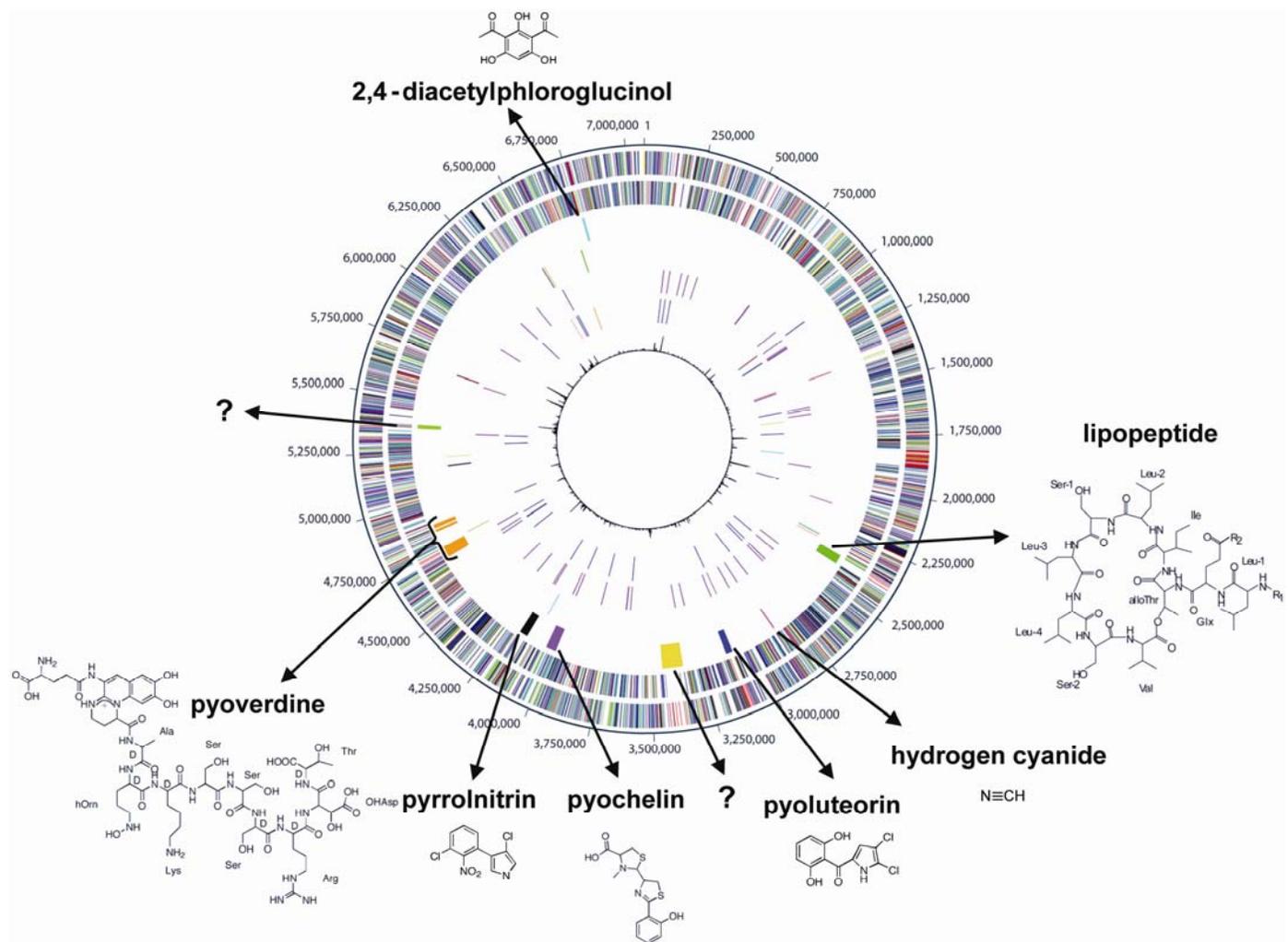


Fig. 1. Circular representation of the genome of *Pseudomonas fluorescens* Pf-5. The outer scale designates coordinates in base pairs, with the origin of replication at 1 bp. The first circle (outermost circle) shows predicted coding regions on the plus strand color-coded by role categories: violet, amino acid biosynthesis; light blue, biosynthesis of cofactors, prosthetic groups, and carriers; light green, cell envelope; red, cellular processes; yellow, DNA metabolism; light gray, energy metabolism; magenta, fatty acid and phospholipid metabolism; pink, protein synthesis and fate; orange, purines, pyrimidines, nucleosides, and nucleotides; olive, regulatory functions and signal transduction; dark green, transcription; teal, transport and binding proteins; gray, unknown function; salmon, other categories; and blue, hypothetical proteins. The second circle shows predicted coding regions on the minus strand color-coded by role categories. The third circle shows nine secondary metabolite gene clusters, with the structures and names of the corresponding metabolite indicated with arrows. Gene clusters whose metabolic products are unknown are designated with a question mark. Genes in the fourth, fifth, and sixth circle are described clockwise from the origin of replication (1 bp) as follows. The fourth circle shows global regulatory genes reported to influence secondary metabolite production by Pf-5 or the related strain *P. fluorescens* CHA0: blue, *rpoN* (σ^{54}); red, *rpoS* (σ^S) and *rsmZ*; purple, *anr*; green, *rsmE*; salmon, *infC*; cyan, *gacA*; olive, *rsmX*; black, *gacS*; gold, *rsmA*; maroon, *lon*; dark gray, *pqqFABC*; orange, *rsmY*; and green, *ptsP*. The fifth circle shows TonB-dependent outer membrane receptors in purple. The sixth circle shows sigma factors: blue, 27 ECF sigma factors; brown, *rpoN* (σ^{54}); red, *rpoS* (σ^S); gold, *algT*; cyan, *fliA*; salmon, *rpoD* (σ^{70}); and orange, *rpoH* (σ^{32}). The seventh circle shows trinucleotide composition in black; regions that differ markedly from the average trinucleotide composition are likely to have been recently acquired in the evolution of Pf-5.

compounds for nutrition, attachment to surfaces, as well as defense from environmental stress and microbial competition.

Catabolism. The genomic sequence of Pf-5 specifies a broad metabolic capacity that is shared with other species of *Pseudomonas* (11) and is consistent with a saprophytic lifestyle in soil. The genome contains genes encoding for utilization of a broad spectrum of organic acids, sugars, and amino acids, including those typically found in seed or root exudates. Although pseudomonads are known to prefer organic acids over carbohydrates as carbon and energy sources (31), the Pf-5 genome has genes for the metabolism of a number of plant-derived carbohydrates such as maltose, sucrose, trehalose, and xylose. Also present are genes for utilization of more complex plant-derived molecules such as the aromatic compounds vanillate, benzoate, and hydroxybenzoate, as well as long chain fatty acids and hydrocarbons, of which many plant oils are comprised. Many of the Pf-5 genes that reflect a “preference” toward plant-derived compounds are shared with *P. syringae* (7,13,19), and may be indicative of its association with plants.

Attachment. The Pf-5 genome contains several genes that have been implicated in the attachment of *Pseudomonas* spp. to surfaces, including genes encoding for predicted hemagglutinins, haemolysins, and other adhesion-related proteins. A subset of these genes is known to be involved in attachment of cells to plant surfaces. For example, Pf-5 has a full complement of genes encoding the biosynthetic pathway for alginate, an exopolysaccharide involved with bacterial attachment to plant surfaces (2), as well as genes involved in the biosynthesis of a type IV pilus, previously demonstrated to have a role in *Pseudomonas* attachment to leaves (57). Pf-5 also contains an *aggA* gene homologue encoding for an agglutinin previously described as a root attachment protein in *P. putida* (6). Espinosa-Urgel et al. (12) characterized several *P. putida* mutants deficient in attachment to corn seeds, which were designated *mus* (mutants unattached to seeds). Many of the *mus* loci also exist in Pf-5. These include the *lapA* gene encoding for a large adhesion protein associated with the *mus-24* mutant (16) and the *hlpBA* genes encoding a two-partner secretion system associated with the *mus-27* mutant (41). Pf-5 also contains other genes associated with attachment, such as the transcriptional factor *adnA* originally identified in *P. fluorescens* Pf0-1 (8,10). *AdnA* mutants of Pf0-1 exhibit decreased adherence to sand and seeds, suggesting that one or more genes belonging to the *adnA* regulon are involved with attachment. It is apparent that several genes related to surface attachment exist in Pf-5, but their functions, especially as they pertain to plant colonization, remain unclear.

Self-defense. Compared with the genomes of *P. aeruginosa*, *P. putida*, or *P. syringae* pv. *tomato*, the genomic sequence of Pf-5 reveals an expanded collection of efflux systems, which typically confer protection against a range of toxic metabolites. For example, multiple drug resistance within *Pseudomonas* spp. has been associated, in part, with efflux systems, specifically those belonging to the resistance-nodulation-cell division (RND) superfamily (46). Within the genome of Pf-5, 13 regions contain RND homologs, with neighboring genes encoding partner proteins of the efflux systems. Other genes are predicted to confer resistance to specific toxins, including tabtoxin, a phytotoxin produced by *P. syringae* pv. *tabaci*, and fusaric acid, a toxin produced by the soilborne plant pathogen *F. oxysporum* that serves as a signal repressing the production of 2,4-diacetylphloroglucinol by *P. fluorescens* CHA0 (43).

Copper is among the chemicals used in agriculture to control plant diseases and, consequently, certain bacterial plant pathogens and soil bacteria have developed resistance to this metal. The *copABCDS* cluster is responsible for copper resistance in the plant pathogen *P. syringae* pv. *tomato* PT23.2 (39). These genes are also present in Pf-5, but they are organized in pairs (*copAB*, *copCD*, and *copRS*) that are dispersed throughout the genome. Pf-

5 exhibits moderate levels of copper resistance, tolerating only 0.32 mM cupric sulfate (M. D. Henkels, C. M. Press, and J. E. Loper, *unpublished data*), which could be related to the dispersed locations of the copper resistance genes in the Pf-5 genome. In contrast, copper-sensitive *P. syringae* DC3000, *P. putida* KT2440, and *P. aeruginosa* PAO1 have homologs of *copAB* but lack *copCDRS* (7,42,64).

The capacity to detoxify active oxygen species is thought to confer a fitness advantage on *Pseudomonas* spp. in the rhizosphere, because mutants of *P. putida* deficient in superoxide dismutase establish smaller population sizes in the rhizosphere than does the parental strain (24). The presence of numerous copies of genes conferring tolerance to oxidative stress (i.e., 10 peroxidases, six catalases, and two superoxide dismutases) in the genome of Pf-5 supports the proposed importance of oxidative stress tolerance to the fitness of this rhizosphere bacterium.

Rhizosphere colonization. *P. fluorescens* Pf-5 was isolated from the cotton rhizosphere and has the capacity to establish populations of sufficient size to suppress certain soilborne pathogens on seed surfaces (17,18). Nevertheless, the population size established by Pf-5 in the rhizosphere of pea or wheat is neither as large nor as persistent as populations established by the most rhizosphere competent strains of *P. fluorescens*, represented by strain Q8r1-96 (27,28). In an attempt to identify genes required for rhizosphere competence, genes present in *P. fluorescens* Q8r1-96 but absent in a less rhizosphere competent strain were identified by subtractive hybridization (35). The putative rhizosphere competence genes identified in that study are not present in Pf-5. The genomic sequence of Pf-5, coupled with detailed knowledge of its biology and the biology of related strains, provides a framework for distinguishing unique genes key to the growth, persistence, and activity of plant-associated bacteria. These studies on rhizosphere competence provide an early example of the value of genomic sequence data in comparative studies of biological control bacteria.

Siderophore-mediated iron acquisition. Due to the limited availability of iron in rhizosphere and bulk soils, plant pathogens are subject to iron-competition by siderophore-producing biological control agents such as Pf-5 (32). Siderophores are high-affinity ferric-iron chelators that are exported from the cell and chelate iron in the extracellular milieu; the iron-siderophore complex is then transported into the cell via specific outer-membrane receptor proteins, thereby providing iron for cellular functions. The Pf-5 genome specifies the biosynthesis of two siderophores—pyoverdine and pyochelin (or related compounds). Genes required for pyoverdine biosynthesis and uptake are organized in three gene clusters in the Pf-5 genome, a pattern shared among certain other *Pseudomonas* spp. (53). A putative pyochelin biosynthesis gene cluster is present in the Pf-5 genome, although its organization differs from the well-characterized pyochelin gene cluster of *P. aeruginosa* (40). *Pseudomonas* spp. are known to utilize siderophores produced by other microorganisms as sources of iron, and genes encoding outer membrane receptors for 20 to 30 heterologous siderophores exist in the genomes of all *Pseudomonas* spp. sequenced to date. Pf-5 is no exception to this, as it has 45 genes predicted to encode outer-membrane proteins that bind the transmembrane protein TonB, a characteristic of ferric-siderophore receptors (Fig. 1). Collectively, these outer membrane receptors should allow Pf-5 to utilize a wide array of siderophores produced by soil microorganisms.

It is quite possible that, when occupying natural habitats on root or soil surfaces, Pf-5 commonly acquires iron by uptake of siderophores produced by its co-inhabitants rather than relying only on the siderophores it produces. This scenario has been demonstrated for a strain of *P. putida*, which does not produce aerobactin and enterobactin but can utilize the ferric complexes of these siderophores as sources of iron. In the rhizosphere, iron availability to *P. putida* is increased in the presence of strains of

Enterobacter cloacae that produce aerobactin and enterobactin (33), indicating that the uptake of heterologous siderophores is an important component of iron nutrition in this natural habitat. In contrast, when grown in isolation on an iron-deficient culture medium, *Pseudomonas* spp. must rely on siderophore production for iron nutrition. Perhaps not coincidentally, siderophore-mediated iron competition between biocontrol strains of *Pseudomonas* spp. and their target pathogens is easily observed on an iron-deficient culture medium in the laboratory, but its occurrence is quite sporadic in natural habitats such as the rhizosphere (32). Similarly, the expression of an iron-regulated promoter in the pyoverdine biosynthesis and uptake gene cluster is higher in culture and in the rhizosphere of plants grown in sterilized soil than in nonsterilized field soil (33). These results, coupled with genomic data indicating the prevalence of siderophore uptake systems in *Pseudomonas* spp., suggest that these bacteria may not produce siderophores consistently on roots of field-grown plants due to the availability of readily utilized siderophores produced by other microorganisms in the environment. If this is the case, then it could explain the discrepancy observed between the role of siderophores in the interactions between *Pseudomonas* spp. and target pathogens on an iron-limited culture medium (where siderophores are reliably produced) versus the rhizosphere.

Secondary metabolites and other secreted products. Approximately 6% of the Pf-5 genome is devoted to the production of secondary metabolites, based upon the sizes of the nine biosynthetic gene clusters identified to date. The nine gene clusters are distributed over a large portion of the genome (Fig. 1). As stated above, six of the gene clusters encode for the biosynthesis of compounds that were known to be produced by Pf-5 before the genomic sequencing project was initiated. Gene clusters for three other secondary metabolites were identified in the genome of Pf-5 based upon characteristic sequences of polyketide or peptide synthases. These enzymes catalyze the formation of secondary metabolites through a nonribosomal mechanism of biosynthesis (66). Although the structures of compounds generated nonribosomally are diverse, the enzymes involved in their biosynthesis have characteristic functional domains. These domains are encoded by highly conserved sequences that can be used to identify biosynthetic gene clusters containing polyketide or peptide synthases. This approach was used to identify three gene clusters in the Pf-5 genome that presumably encode for secondary metabolites. For one peptide synthase, the amino acid sequence of the synthesized product was predicted based upon the sequence specificity of each functional domain in the enzyme for a particular amino acid in the elongating peptide chain (47). Subsequently, the structure was elucidated by purifying the compound from cultures of Pf-5 and subjecting it to chemical analysis, thereby confirming the amino acid sequence predicted using bioinformatics (H. Gross, J. E. Loper, and W. Gerwick, *unpublished data*). The three metabolites discovered through genomic sequencing have not yet been characterized with respect to their toxicities to plant pathogens or their roles in biological control. Nevertheless, their discovery provides new directions for research evaluating mechanisms of biological control.

In addition to secondary metabolites, Pf-5 produces other exported products, including exoenzymes and at least one bacteriocin. Many of these products, such as an extracellular alkaline protease that suppresses the root knot nematode *Meloidogyne incognita* (62), have been recognized characteristics of Pf-5 for many years. In contrast, bacteriocin production by Pf-5 has not been characterized until recently, when the detection of two homologs of *llpA* in the Pf-5 genome resulted in research demonstrating that this strain produces a bacteriocin related to LlpA (45).

Regulatory circuitry. As expected for a bacterium with a large genome that lives in a rapidly changing environment, Pf-5 has an

extensive collection of regulatory genes. Among the proteobacteria whose genomes have been sequenced, Pf-5 has the largest number of sigma factors in the extracytoplasmic factor (ECF) class (22). Genes for 21 of the 27 ECF sigma factors in the Pf-5 genome are in clusters also containing genes encoding TonB-dependent outer-membrane receptors (Fig. 1). A smaller number of such gene clusters also have been reported in the genomes of other *Pseudomonas* spp. where, in response to a specific ferric-siderophore complex, the ECF sigma factor controls expression of the adjacent receptor gene (50). The predicted 68 histidine kinases and 113 response regulators in the Pf-5 genome also exceed the number predicted for any of the other *Pseudomonas* spp. whose genomic sequences are published (23). Global regulatory genes that control secondary metabolite production by Pf-5 or the related strain CHA0 are widely distributed in the genome of Pf-5 (Fig. 1), which is not unexpected given the broad influences of these genes on the physiology of the bacterium. It is likely that many of the regulatory genes identified through the genomic sequencing of Pf-5 will also play a role in secondary metabolite production and biological control.

Lack of key pathogenicity factors. Consistent with its commensal lifestyle, Pf-5 lacks a number of virulence factors found in plant pathogens. No evidence for a type III secretion system was found in the genomic sequence of Pf-5, although genes for these export systems have been found in other nonpathogenic strains of *Pseudomonas* spp. (including *P. fluorescens*) associated with plants (36,51,55). There is no evidence in the Pf-5 genome for the biosynthesis of the known *P. syringae* phytotoxins tabtoxin, syringomycin, syringotoxin, syringopeptin, or coronatine. Pf-5 lacks amylase, consistent with an inability to utilize starch, and lacks cellulases and other exoenzymes often associated with degradation of plant cell walls and cell wall components. Therefore, many genes required for pathogenicity or virulence of plant or animal pathogens do not have clear counterparts in the genome of this commensal bacterium.

Perspectives and future directions. As discussed throughout this review, bioinformatic analysis of the Pf-5 genome has led to the development of hypotheses regarding aspects of biological control that can now be further studied experimentally. Examples presented herein include the discovery of new secondary metabolites, possibly with antibiotic activities; new approaches to unravel key processes in rhizosphere colonization; and the opportunity to greatly expand our knowledge of factors regulating key biocontrol traits. Also discussed was a possible explanation for the infamous disparity between the success of biological control in a defined environment versus in a field soil, using siderophore-mediated biological control as an example. Comparative genomics has already highlighted important distinctions between Pf-5 and pathogenic *Pseudomonas* spp. In the future, such comparisons will be invaluable in identifying genes and functions that are shared or unique to the commensal or pathogenic lifestyles of bacteria in the phytosphere. The availability of the genome sequence of Pf-5 also opens the door to enable high throughput "hypothesis-generating" approaches to investigate biological control, such as whole genome microarrays for transcriptional profiling, proteomic approaches such as mass spectrometry-based methods for studying protein abundances and interactions, and large-scale gene knockout or heterologous expression studies. As the genomic sequences of more biological control agents become available and functional and comparative genomics of these organisms progress, we expect that genomics will become an increasingly important component of the scientific foundation underpinning the biological control of plant disease.

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