

# Effect of Tannic Acid on the Transcriptome of the Soil Bacterium *Pseudomonas protegens* Pf-5

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**Tannins are a diverse group of plant-produced, polyphenolic compounds with metal-chelating and antimicrobial properties that are prevalent in many soils. Using transcriptomics, we determined that tannic acid, a form of hydrolysable tannin, broadly affects the expression of genes involved in iron and zinc homeostases, sulfur metabolism, biofilm formation, motility, and secondary metabolite biosynthesis in the soil- and rhizosphere-inhabiting bacterium *Pseudomonas protegens* Pf-5.**

Tannins are polyphenolic compounds produced in the leaves, roots, bark, galls, fruits, and buds of many plants (1). Tannins can be divided into two types: condensed and hydrolysable. Condensed tannins are made up of flavonol polymers (2), whereas hydrolysable tannins are composed of a central polyol, esterified to gallic acids to form gallotannins (3). From these basic structures, plants synthesize many derivatives with diverse functions, including defense against herbivores and pathogens (4). An important property of tannins is their ability to form complexes with metals such as iron (5, 6), copper (7), and zinc (8, 9). Tannins can also bind proteins (10), scavenge free radicals (1), and inhibit microbial growth (4, 11, 12), possibly through tannin-polymer complexation, membrane disruption, and/or chelation of metal ions (13).

Tannins are among the most abundant organic compounds in plants, but knowledge of their concentrations in soil is vague due to differences in physical, chemical, and biotic properties of the soils and in methods for tannin quantification (14–16). Estimated concentrations of phenolic compounds in soil and humus vary between 0.18 and 37.6 mg/g dry weight (17–20). At these concentrations, tannins are likely to affect the physiology of microorganisms inhabiting the soil or rhizosphere.

Here we describe the effect of tannic acid (TA), a form of hydrolysable tannin, on the transcriptome of the model biocontrol bacterium *Pseudomonas protegens* Pf-5 (previously called *Pseudomonas fluorescens* Pf-5) (21, 22). *P. protegens* Pf-5 was isolated from soil and can colonize root and seed surfaces (23), protecting them from fungal, oomycete, and bacterial pathogens, primarily through the secretion of a range of bioactive secondary metabolites and exoenzymes (24, 25). Given the wide distribution of tannins in soil and their high abundance in some roots and seeds (26, 27), these compounds could have an important influence on the gene expression and secondary metabolism of soil and rhizosphere bacteria such as Pf-5. To date, few studies have investigated the role of plant-derived phenolic compounds in gene expression by biocontrol bacteria (28, 29).

In Mueller-Hinton (MH) broth (Oxoid, Thermo Fisher Scientific, Adelaide, SA, Australia), TA (Sigma-Aldrich, St. Louis, MO) inhibited the growth of *P. protegens* Pf-5 at concentrations exceeding 20  $\mu\text{g/ml}$  (Fig. 1). Concentrations of TA above 300  $\mu\text{g/ml}$  also influenced cell morphology, inducing filament formation (see Fig. S1 in the supplemental material), as observed previously in *P. fluorescens* (30). Experiments evaluating the Pf-5 transcriptome

were done in MH amended with two TA concentrations: 20  $\mu\text{g/ml}$  (low TA), which did not significantly affect growth, and 160  $\mu\text{g/ml}$  (high TA), which resulted in significantly lower cell density (Fig. 1).

The transcriptomic effects of TA were determined using whole-genome microarrays as described previously (31, 32). Amendment of MH medium with TA had a broad influence on the Pf-5 transcriptome, with the transcript abundance of 64 genes altered significantly, by at least 2-fold, at the low TA concentration and 575 genes at the high TA concentration (Fig. 2; see also Table S1 in the supplemental material). The low TA concentration induced expression of many genes in two functional-role categories, transcription and central intermediary metabolism, whereas the high TA concentration significantly affected genes in 19 of the 24 role categories (see Fig. S2 in the supplemental material). Quantitative reverse transcriptase PCR (qRT-PCR) validation was performed with a set of gene-specific primers as described previously (31) (see Table S2 in the supplemental material), and the results correlated highly with the microarray data (see Fig. S3 in the supplemental material).

TA had a strong effect on the transcription of genes involved in iron homeostasis of Pf-5, enhancing the expression of genes encoding heme uptake and biosynthesis and transport of the siderophores pyoverdine and enantio-pyochelin (33, 34). Pyoverdine production by Pf-5 also increased in a dose-dependent manner in response to TA (see Fig. S4 in the supplemental material). Genes having putative roles in iron storage (such as PFL\_4769, PFL\_4859, and PFL\_5555) were downregulated, whereas PFL\_4858, which encodes a bacterioferritin-associated ferredoxin that mobilizes iron stored in bacterioferritin B (35), was upregu-

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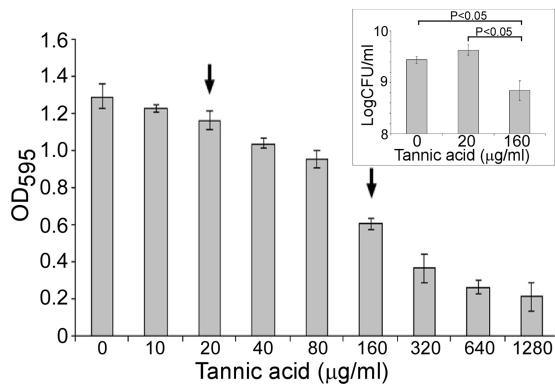
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**FIG 1** Effects of TA on the growth of *Pseudomonas protegens* Pf-5. Growth of Pf-5, assessed by measuring the optical density at 595 nm (OD<sub>595</sub>), in MH medium containing various concentrations of TA at 25°C for 2 days. Arrows depict TA concentrations used in the transcriptomic studies. Error bars denote standard deviations. (Inset) Effects of TA on growth of Pf-5 assessed as CFU per ml after plating on Luria-Bertani agar plates containing streptomycin (100 µg/ml). Statistical analysis was performed using a *t* test ( $P \leq 0.05$ ). No statistically significant difference in growth of Pf-5 was observed between the non-amended and low-TA-amended (20 µg/ml) cultures. Concentrations of TA did not significantly alter the pH of the spent culture medium (control, pH 7.06; low-TA treatment, pH 7.01; and high-TA treatment, pH 6.99).

lated by TA. These effects are probably due to the iron-sequestering capacity of TA, leading to lowered iron bioavailability in the medium amended with TA, especially at the high concentration. Indeed, significant overlap was observed between the transcriptional profiles of Pf-5 grown in TA-amended medium and in an iron-limited medium evaluated previously (see Table S1 in the supplemental material) (36) and many genes downstream from putative ferric uptake regulator (Fur) binding sites (32) were upregulated in the TA-amended medium (see Table S1).

By reducing the levels of available iron, TA can diminish the formation of reactive oxygen species via the Fenton reaction, thereby reducing oxidative stress (6, 37, 38). TA may also scavenge HO radicals through its many phenolic groups that act as efficient nucleophiles (39, 40). In Pf-5, the transcription of genes involved in the oxidative stress response, such as *katA* and *katG*, was downregulated in the high-TA medium (see Table S1 in the supplemental material).

In addition to iron, TA can chelate other metals (7, 41), including zinc, and TA modulated the expression of several genes in Pf-5 that are also regulated by zinc (31). For example, TA induced the expression of PFL\_0078, which encodes the putative zinc uptake regulator Zur (42), and of a number of genes located downstream of putative Zur binding sites (see Table S1 in the supplemental material). Among these is PFL\_4896, which encodes the zinc-independent form of ribosomal protein L31 (C<sup>-</sup> form). In contrast, the transcript levels of PFL\_0441, which encodes the zinc-binding paralog (C<sup>+</sup> form), were not influenced significantly by TA. The commonalities between the influences of high TA and low zinc on the Pf-5 transcriptome suggest that TA may affect zinc homeostasis.

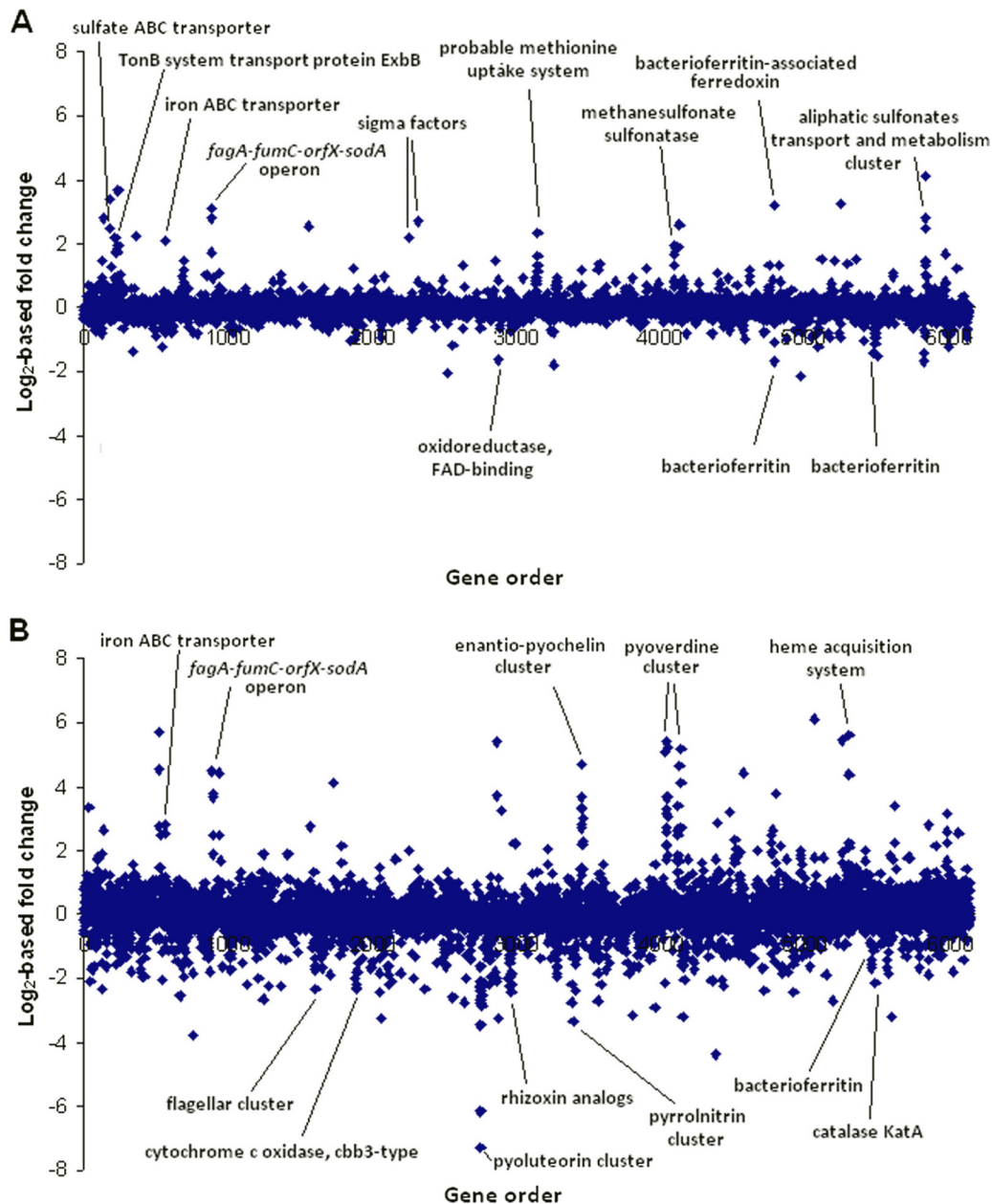
TA treatments also perturbed the transcription of genes involved in respiration. Pf-5 possesses a highly branched respiratory chain with multiple terminal oxidases (22), which provide respiratory flexibility (43). Genes for the cytochrome *cbb*<sub>3</sub>-2 oxidase, which requires iron as a cofactor, were downregulated in the

high-TA medium and in the iron-limited medium evaluated previously (see Table S1 in the supplemental material) (36). In contrast, *cyoABCDE*, which encode a cytochrome *bo*<sub>3</sub> quinol oxidase, were upregulated in both TA-containing and iron-limited media (36). In *Pseudomonas aeruginosa*, this terminal oxidase has a lower requirement for iron than its counterparts (43). Thus, the altered transcription of the alternative terminal oxidases of Pf-5 in response to high TA concentration could be due, at least in part, to reduced bioavailability of iron in the medium.

Several genes upregulated by TA could contribute to TA resistance. For example, the *lrgAB* operon, upregulated by high TA, may encode an antiholin-like protein (44) involved in murein hydrolase activity, oxidative stress, and penicillin tolerance (45, 46). In *Staphylococcus aureus*, these proteins may participate in the cell wall stress response (47), and a study with *Lactobacillus plantarum* indicated that the cell wall might be a site of action by TA (48). The MdtC-like RND efflux gene was upregulated under TA stress. The MdtABCD efflux system was also overexpressed in *Escherichia coli* after treatment with condensed tannin (49), suggesting that it may act as an efflux system for tannins. PFL\_1592 was also overexpressed in response to TA. The homologous gene (29% amino acid identity) in *Escherichia coli*, which encodes Spy (spheroblast protein y), was highly expressed in response to condensed tannin (49, 50). The Spy protein is postulated to be a chaperone that helps to protect cells from protein aggregation and inactivation as a result of tannin treatment (50) and may therefore constitute an important resistance factor.

A number of gene clusters involved in sulfur metabolism and transport were upregulated in cultures of Pf-5 grown in MH medium amended with low TA concentrations (see Table S1 in the supplemental material). Thirty-seven genes upregulated by low TA have orthologs in *P. aeruginosa* PAO1, and of those, 15 orthologs were overexpressed in sulfate-starved cells of *P. aeruginosa* in a previous study (see Table S1) (51). The high TA concentration also influenced the transcript levels of many genes in Pf-5 that are orthologous to genes regulated by sulfate starvation in *P. aeruginosa*, but few of these sulfur-related genes were regulated by both low and high TA concentrations. The differential effects of the two TA concentrations on the expression of sulfur-related genes may be indicative of strict regulation of sulfur metabolism in Pf-5, which is likely to be influenced by the concentration of sulfur and other nutritional factors, such as iron homeostasis, that are influenced by TA.

Pf-5 is a model biocontrol strain, so the effect of TA on the expression of known biocontrol factors was of particular interest. Under high TA, several gene clusters involved in bioactive secondary metabolite biosynthesis or exoenzyme production were downregulated, including those for pyoluteorin, pyrrolnitrin, rhizoxin analogs, the extracellular protease AprA, and chitinase. Genes encoding bacteriocins that function in bacterial competition (52) were also downregulated. These results extend previous studies demonstrating that many plant-derived phenolic compounds influence the expression of genes involved in 2,4-diacetylphloroglucinol and pyoluteorin production in *P. protegens* CHA0 (28, 29), which is closely related to Pf-5. The mechanism(s) by which TA influences the expression of biocontrol factors in Pf-5 is unknown but could be related to stress responses of the cell, as coordinated expression of genes involved in stress responses, secondary metabolism, and exoenzyme production is well established in *P. protegens* (53, 54). In support of this possibility, only the high TA con-



**FIG 2** Influence of TA on the transcriptome of *Pseudomonas protegens* Pf-5. Differential gene transcription of strain Pf-5 grown in MH medium (to an  $OD_{600}$  of approximately 1.3) amended with a low TA concentration (20  $\mu\text{g/ml}$ ) (A) or a high TA concentration (160  $\mu\text{g/ml}$ ) (B) versus that in the nonamended medium. Each dot represents one of the genes in the Pf-5 genome, with the  $x$  axis showing gene order with the origin of replication situated at both ends. The  $y$  axis shows the  $\log_2$  of the fold change of each gene in the TA-amended medium versus the nonamended medium. The microarray data were stored in the Gene Expression Omnibus (GEO) database (accession number GSE33909).

centration, which exhibited some toxicity to Pf-5, significantly influenced the transcript levels of the secondary metabolite biosynthesis, exoenzyme, and bacteriocin genes.

In the medium amended with the high TA concentration, numerous genes related to biofilm formation and stability in Pf-5 were downregulated, including putative poly- $\beta$ -1,6-*N*-acetyl-D-glucosamine (PGA) biosynthesis genes (55) and the *psl*ABDEFGHIJ cluster (56). In flow cells, biofilm formation by Pf-5 was reduced by at least 30-fold after 72 h in the high-TA medium versus the non-TA-amended control (see Fig. S5 in the supplemental material). Previ-

ously, TA has shown effects on biofilm formation in other bacterial species (57, 58).

TA reduced the swarming motility of Pf-5 in a dose-dependent manner (see Fig. S6 in the supplemental material), which is consistent with the observation that condensed or hydrolysable tannins reduce swarming of *P. aeruginosa* (59). The high TA concentration downregulated many genes with putative roles in motility, including genes conferring flagellar biosynthesis (see Table S1 in the supplemental material) and those encoding methyl-accepting chemotaxis acceptors (see Table S3 in the supplemental material).

Increasing the TA concentration also caused filamentation and aggregation of cells (see Fig. S1 in the supplemental material) and reduced the growth of Pf-5 (Fig. 1), which could also contribute to the reduced swarming motility observed. However, the swarming motility of Pf-5 was also reduced by the low TA concentration (see Fig. S6), which caused no reduction in cell density (Fig. 1). In previous studies, we observed that iron limitation could reduce the swarming motility of Pf-5 (36). Therefore, depletion of iron in the media by TA could cause the inhibition of the swarming motility of this bacterium. Alternatively, tannins could bind to bacterial structures that are involved in motility. For example, an outer membrane-associated lipopolysaccharide of *P. aeruginosa* that is required for swarming (60) can be bound by condensed tannins (59, 61) and there is the possibility that flagellin subunits can be bound by TA as well (59, 62).

In conclusion, we demonstrated that the plant-derived polyphenol TA has broad effects on the transcriptome of the biocontrol bacterium *P. protegens* Pf-5. Genes related to iron and zinc homeostases, as well as stress responses, were modulated in the presence of TA. These effects could be due to the ability of TA to bind free metals or scavenge free radicals. In addition, TA treatment reduced the transcription of genes for secondary metabolite biosynthesis and exoenzyme and bacteriocin production, as well as motility and biofilm formation. The products or traits from these genes are thought to contribute to biological control and rhizosphere colonization by *Pseudomonas* spp. (63, 64). These results extend previous studies indicating that low-molecular-weight phenolic compounds influence the expression of antibiotic biosynthesis genes by *P. protegens* strain CHA0 (28, 29) and that tannins influence iron availability to *Pseudomonas syringae* (65). Taken together, these studies suggest that plant-derived phenolics have a broad influence on the physiology and behavior of plant-associated bacteria.

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