

## *Rhizobium etli* USDA9032 Engineered To Produce a Phenazine Antibiotic Inhibits the Growth of Fungal Pathogens but Is Impaired in Symbiotic Performance<sup>∇</sup>

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**Phenazine production was engineered in *Rhizobium etli* USDA9032 by the introduction of the *phz* locus of *Pseudomonas chlororaphis* O6. Phenazine-producing *R. etli* was able to inhibit the growth of *Botrytis cinerea* and *Fusarium oxysporum* in vitro. Black bean inoculated with phenazine-producing *R. etli* produced brownish Fix<sup>-</sup> nodules.**

Several root-colonizing *Pseudomonas* spp. are potent biocontrol agents (7, 10, 16). *Pseudomonas* spp. produce phenazine antibiotics, nitrogen-containing heterocyclic pigments, which exhibit broad-spectrum activity against numerous bacteria and fungi. More than 50 naturally occurring phenazine compounds have been reported, with some of the *Pseudomonas* spp. synthesizing a mixture of phenazine derivatives (18). *Pseudomonas chlororaphis*, a plant growth-promoting rhizobacterium, produces phenazine antibiotics that exhibit antifungal activity (1, 2, 5, 17). The phenazine biosynthetic locus consists of seven genes (*phzABCDEFG*) arranged in a single operon (3, 12, 14). In spite of the potential of plant-growth-promoting rhizobacteria as a potent biocontrol agent against plant diseases, field tests have revealed inconsistent results (4).

Soil-bacteria belonging to the genera *Allorhizobium*, *Azorhizobium*, *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium*, collectively known as rhizobia, have the unique ability to form specialized structures called nodules on compatible legume roots or stems. Because of their ubiquitous occurrence and biological nitrogen-fixing ability, these microsymbionts can be exploited as potential biocontrol agents if they can be engineered to produce antibiotics, such as phenazine. Phenazine synthesis, however, occurs in specific bacterial genera, including *Pseudomonas*, *Burkholderia*, *Brevibacterium*, and *Streptomyces*, but not in rhizobia (18). Here, we report the expression of a phenazine biosynthetic operon of *Pseudomonas chlororaphis* O6 in *Rhizobium etli* USDA9032.

A pLAFR1 cosmid clone (pO6phz), which carries the phenazine biosynthetic genes of *P. chlororaphis* O6, was introduced into *R. etli* USDA9032 by triparental mating with pRK2013 as the helper plasmid (6). Phenazine production by the *R. etli* strain in vitro was assayed by using yeast extract mannitol (YEM) broth. Rhizobia were grown in a reciprocal shaker at

180 rpm for 72 h at 30°C. The samples were extracted twice with an equal volume of acidified ethyl acetate, dried under a fume hood, and resuspended in 1 ml of 1 mM NaOH (9). The absorbance of the extract was monitored spectrophotometrically. Phenazine absorption spectra exhibit a characteristic peak at 365 nm (13). Mobilization of the *phz* locus of *P. chlororaphis* O6 into *R. etli* USDA9032 resulted in the production of a characteristic absorption peak at 365 nm, indicative of phenazine production. Thin-layer chromatography analysis with Silica Gel G chromatography plates in an ascending benzene/acetic acid mixture (95:5, vol/vol) demonstrated that the *R. etli* carrying the *phz* genes produced a yellow-colored compound that had an  $R_f$  value similar to that for 2-hydroxyl phenazine 1-carboxylic acid produced by *P. chlororaphis* 30-84 (Fig. 1). In addition, *P. chlororaphis* O6 produced compounds with  $R_f$  values similar to those for 2-phenazine carboxylic acid and 2-hydroxyl phenazine. These secondary metabolites were not detected in the *R. etli* transconjugant carrying the cloning vector (Fig. 1).

To verify whether phenazine-producing *R. etli* has acquired the ability to suppress fungal growth, in vitro fungal inhibition assays were performed. *P. chlororaphis* O6, which produces phenazine antibiotics, was effective in suppressing the growth of *Botrytis cinerea* and *Fusarium oxysporum* in a plate assay. Interestingly, phenazine-producing *R. etli* transconjugants also were able to inhibit the fungal growth (Fig. 2). In contrast, *R. etli* carrying the cloning vector alone had no effect on the fungal growth (Fig. 2).

To determine the consequences of phenazine production on symbiotic performance, we inoculated a host plant with phenazine-producing *R. etli*. Nodulation responses were assessed at 15 and 25 days after inoculation. The acetylene reduction assay was performed by the method of Schwinghamer et al. (15). Black bean inoculated with a phenazine-nonproducing strain appeared healthy and produced dark green leaves. Plants inoculated with phenazine-producing strain produced leaves that were pale green, indicative of nitrogen deficiency. Roots of these plants revealed numerous small nodules that were

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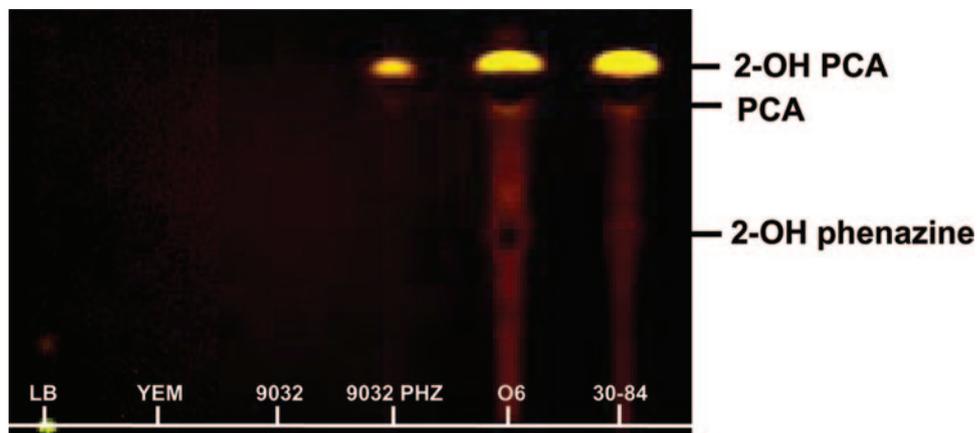


FIG. 1. Detection of phenazine in *Rhizobium etli* and *Pseudomonas chlororaphis*. Phenazine antibiotics extracted in benzene were analyzed by using thin-layer Silica Gel G chromatography plates in an ascending benzene/acetic acid mixture (95:5, vol/vol). Individual phenazine compounds, 2-hydroxyphenazine-1-carboxylic acid (2-OH PCA), phenazine-1-carboxylic acid (PCA), and 2-hydroxyphenazine (2-OH phenazine) were visualized by UV irradiation at 365 nm. LB, Luria-Bertani broth; YEM, YEM broth; 9032, *R. etli* 9032 containing pLAFR1; 9032 PHZ, *R. etli* 9032 carrying the phenazine biosynthetic genes; O6, *P. chlororaphis* O6; 30-84, *P. chlororaphis* 30-84.

brownish in color. These nodules exhibited no detectable acetylene reduction activity. An examination of the acidified ethyl acetate extract from the nodules under long-wave UV irradiation revealed a peak at 365 nm, indicating the production of phenazine inside the nodules (data not shown). The nodules initiated by the *R. etli* (pLAFR1) were pink in appearance, with characteristic ridges on the surface.

The ultrastructure of the nodules initiated by phenazine-producing and -nonproducing strains of *R. etli* was examined by transmission electron microscopy (Fig. 3). A low-magnification view of cells from the infected region of nodules initiated by *R. etli* (pLAFR1) showed numerous bacteroids, which were enclosed by the peribacteroid membrane (symbiosome). Predominantly, the symbiosomes enclosed one or two bacteroids (Fig.

3B). Nodules initiated by the phenazine-producing strain contained cells with bacteroids that were enclosed by a dilated peribacteroid membrane having a vacuolar appearance (Fig. 3C). These dilated sacs often enclosed more than two bacteroids. In contrast to the wild-type bacteria, phenazine-producing bacteria contained numerous prominent polyhydroxybutyrate inclusions (Fig. 3C). An additional anatomical feature of these nodules was the presence of numerous mitochondria, some of which with unusual shapes (Fig. 3D).

To ascertain whether phenazine production had deleterious effects on *R. etli*, we examined the growth of rhizobia in broth cultures. *R. etli* harboring the cosmid vector grew normally in YEM broth and reached an optical density of 2.00 after 96 h of growth at 30°C. The phenazine-producing strain grew poorly

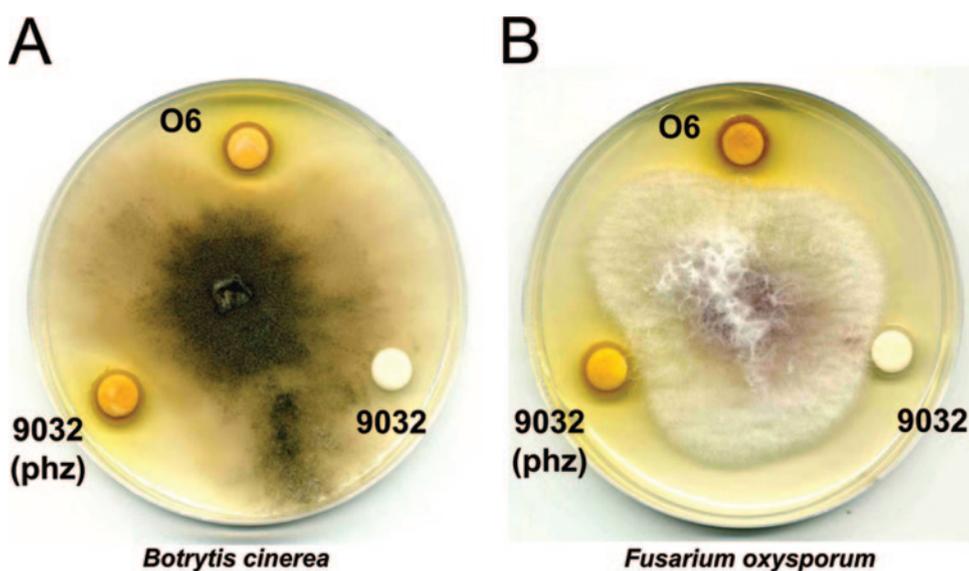


FIG. 2. Fungal inhibition by phenazine-producing *Rhizobium etli*. *Botrytis cinerea* (A) and *Fusarium oxysporum* (B) mycelial plugs were placed at the center and the test strains were placed at the periphery of the plate, which was incubated at 30°C. *Rhizobium etli* (phz) and *P. chlororaphis* O6 showed inhibition of fungal mycelial growth. 9032, *R. etli* 9032 containing pLAFR1; 9032 PHZ, *R. etli* 9032 carrying the phenazine biosynthetic genes; O6, *P. chlororaphis* O6.

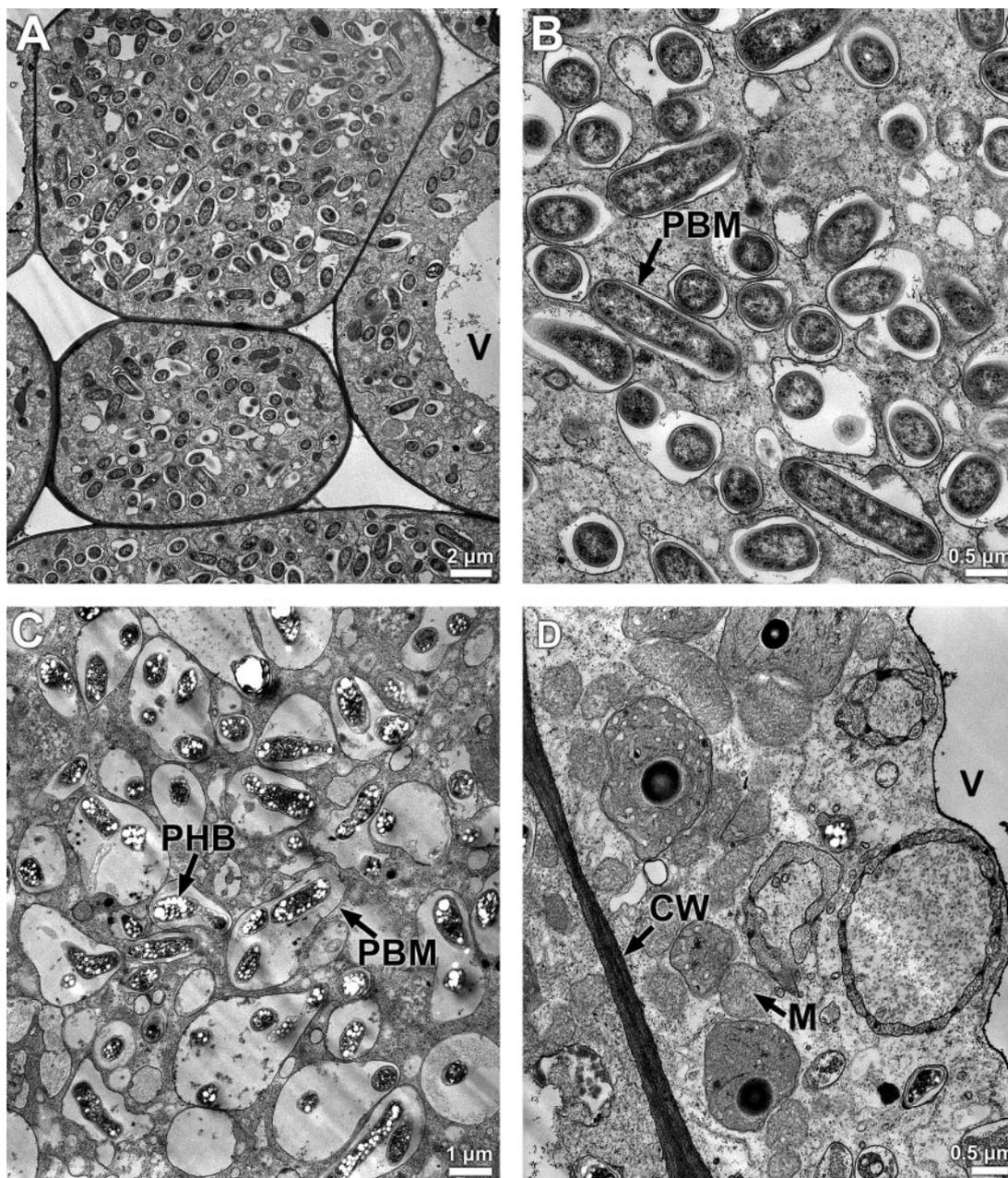


FIG. 3. Transmission electron micrographs of bean nodules. Nodules initiated by wild-type *R. etli* contain cells that are packed with bacteroids (A), which are surrounded by a peribacteroid membrane (B). In contrast, the cells from the nodules formed by a phenazine-producing strain contain bacteria that are enclosed in large sac-like structures (C). Numerous tubular structures and mitochondria of various shapes and sizes are also seen (D). Note the presence of large amounts of poly- $\beta$ -hydroxybutyrate inclusions in phenazine-producing bacteria (C). CW, cell wall; M, mitochondria; V, vacuole; PBM, peribacteroid membrane; PHB, poly- $\beta$ -hydroxybutyrate.

and reached a maximum optical density of 0.7 at the end of the 96-h growth period (Fig. 4). When colonies from 96-h cultures of the phenazine-producing strain were plated, only a few colonies were recovered, indicating that phenazine production inhibited the viability of these cells (data not shown).

Even though we were successful in generating phenazine-producing rhizobia, their utility as a new biocontrol agent against plant pathogens appears limited. Two major deleterious effects of phenazine production in rhizobia were noted: abolition of nitrogen-fixation and inhibition of bacterial

growth. These two problems need to be overcome before phenazine-producing rhizobia could be utilized as a biocontrol agent. The inability of phenazine-producing rhizobia to produce nitrogen-fixing nodules appears to be related to phenazine-mediated membrane damage. It is not known if the membrane damage is the result of a direct or indirect effect of phenazine toxicity. Transmission electron micrographs of nodules initiated by phenazine-producing rhizobia clearly reveal the loss of membrane integrity. Phenazine compounds undergo rapid oxidation and reduction, resulting in the generation of

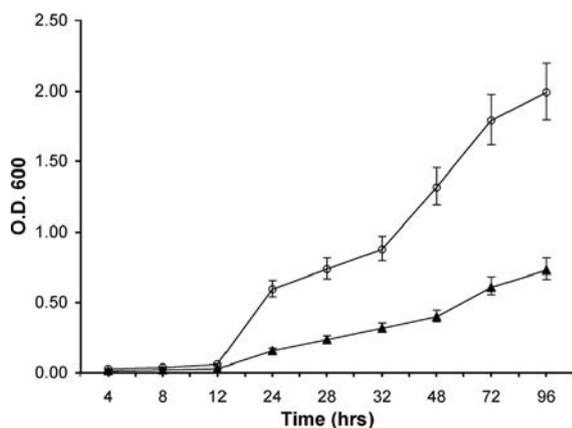


FIG. 4. Growth curves of wild-type (○) and phenazine-producing (▲) *R. etli*. Rhizobia were grown at 30°C in YEM broth, and the optical density at 600 nm (O.D. 600) was measured at various time periods.

free radicals (8). These free radicals are presumably involved in the loss of membrane integrity and consequent cell death. Our observations are consistent with the role of reactive oxygen species in nodule senescence. In senescing nodules, the enhanced production of reactive oxygen species, leading to oxidative damage of macromolecules, has been observed (11).

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The use of product names is necessary to report factually on available data; however, the University of Missouri and USDA neither guarantee nor warrant the standard of the product, and the use of the name by the University of Missouri or USDA implies no approval of the product to the exclusion of others that may be suitable.

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