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**ROOT COLONIZATION BY *PSEUDOMONAS PUTIDA*:  
THE ROLE OF CATALASE AND  
SUPEROXIDE DISMUTASE ISOZYMES**

**by**

**Tawnya Lynn Bowles**

**Thesis submitted in partial fulfillment  
of the requirements for the degree**

**of**

**UNIVERSITY HONORS  
WITH DEPARTMENT HONORS**

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**Biology**

**UTAH STATE UNIVERSITY  
Logan, UT  
1996**

# **Root Colonization by *Pseudomonas putida*: The Role of Catalase and Superoxide Dismutase Isozymes**

*Tawnya L. Bowles, Senior Thesis, Department of Biology, Utah State University, 1996*  
*Thesis Advisory Committee: Anne J. Anderson and Charles Miller*

## **I. GENERAL INTRODUCTION**

The fluorescent soil bacterium *Pseudomonas putida* is an aggressive root colonizer (1) that offers benefits to the host plant by suppressing soilborne pathogens and enhancing plant growth (12,13). Upon introduction in the field through seed inoculation, certain isolates of *P. putida* have the potential to act as an effective biological control agent (3). *P. putida* has also been shown to promote the growth of potatoes, sugarbeets, and radishes (13). Fluorescent pseudomonads are effective in protecting plants against pathogens such as *Gaeumannomyces graminis* var. *tritici*, a fungus that causes the root disease of wheat called take-all (13).

Saprophytic pseudomonads like *P. putida* colonize the surface of the plant root, called the rhizoplane, as well as the zone of soil adjacent to the root, termed the rhizosphere (13). The interactions between the bacteria, the root, the soil environment, and the indigenous microbiota are complex (3). To clarify the role of *P. putida* as a biocontrol agent, the mechanisms of root colonization by this bacterium must be more completely understood.

The ability of *P. putida* to protect itself from the activated oxygen species released from plant roots may be important in effective root colonization (6). Enzymes on the surface of plant roots produce hydrogen peroxide ( $H_2O_2$ ) and superoxide anion ( $O_2^-$ ). These activated oxygen species are harmful to all organisms (6). The enzyme superoxide dismutase (SOD) dismutates  $O_2^-$  to hydrogen peroxide and molecular oxygen ( $O_2$ ) and the enzyme catalase converts  $H_2O_2$  to oxygen and water (4). Bacteria with these enzymes may be more effective at colonizing plant roots because they are able to convert the toxic species into innocuous compounds of water and oxygen (4). Similarly, mammalian bacterial pathogens that possess catalase and

SOD are able to resist the deleterious effects of  $H_2O_2$  and  $O_2^-$  produced by white blood cells in the bloodstream and successfully parasitize their host (4).

*P. putida* has been shown to produce three distinct forms of catalase, called catalase A, B, and C (5,6). Catalase A is known to be cytoplasmic while catalase B is associated with the cell membrane (7). Although the isozymes differ in optimum pH range and response to inhibitors, both appear to be heme proteins because cyanide mutually inhibits their activities (7). The differences in optimal pH and inhibition by certain chemicals suggest that catalase A and B are products of different genes (7). Recent molecular evidence confirms this fact (8).

Previous experiments (5) have monitored the catalase activity of *P. putida* cells exposed to  $H_2O_2$  treatments. Cells in the stationary growth phase were found to be more resistant than logarithmic cells to the toxic effects of  $H_2O_2$  (5). Normally catalase A is expressed in logarithmic cells while isozymes A and B are apparent in stationary cells. However, when logarithmic cells were pretreated with a sublethal amount of  $H_2O_2$ , increased catalase activity and induction of catalase B and C isozymes were observed (5).

It is possible that external  $H_2O_2$  at the plant root may also influence the activity of the *P. putida* catalase enzyme and the isozymes formed (6). When *P. putida* cells in contact with bean roots were examined at various time points, their catalase activity was found to initially decrease but then increase after 12 hours. This increase in catalase activity was associated with the expression of two isozymes, A and B. SOD activity also varied with time, increasing initially and remaining elevated over 48 hours (6). These experiments suggest that regulation of catalase and SOD activities may be necessary for *P. putida* to effectively colonize roots (6).

To further examine the role of catalase and superoxide dismutase isozymes in *P. putida* root colonization, I have compared the extent of bean and wheat root colonization by the *P. putida* wildtype with colonization by catalase- or SOD-deficient mutants. Colonization by a catalase A-deficient mutant (J-1) is discussed in Section II, colonization by a catalase C-deficient mutant is discussed in Section III, and



colonization by a SOD-deficient mutant is presented in Section IV. An overall discussion is presented in Section V. Based on the findings of previous studies, I predicted that mutant isolates would be more vulnerable to the activated oxygen species produced by the plant roots and therefore less efficient root colonizers than the wildtype isolate.

## II. ROOT COLONIZATION BY A CATALASE A-DEFICIENT MUTANT

### INTRODUCTION

To examine the role of catalase A in root colonization by *P. putida*, surface sterilized bean and wheat seeds were inoculated with either the catalase A-deficient mutant, called J-1, or the wildtype strain and planted in sterile vermiculite. The J-1 mutant was formed through chemical mutagenesis with ethyl methanesulfonate (EMS) (1). Root colonization was analyzed at two time points by rinsing roots to release bacterial cells and plating a dilution series of the root wash onto medium to support bacterial growth. Root washes and dilution series were also performed for a sample of sterilized but uninoculated seeds.

### MATERIALS AND METHODS

**Preparation of Cultures.** *P. putida* *nal<sup>R</sup>* isolate Corvallis and a catalase A-deficient mutant (J-1) formed by chemical mutagenesis with ethyl methanesulfonate (EMS) (1) were stored at -80°C in 15% glycerol (50 µg/mL). For culture, cells were transferred into liquid LB media and grown to stationary phase (24-30 hrs).

#### **Sterilization of Seeds.**

##### *Dark Red Kidney Bean Seed Sterilization*

Seeds were soaked in sterile water for 10 min, rinsed, and soaked in 10% sodium hypochlorite with 0.2 % Tween-20 for 10 min. Using sterile water, seeds were rinsed and soaked for 10 minutes before sorting and discarding all seeds without intact seed coats. The above steps were repeated.

##### *Malcolm Wheat Seed Sterilization*

Seeds were subjected to a heat treatment of 40 min at 50°C with vigorous shaking followed by three

sterile water washes (at 50°C). The process was repeated twice. The seeds were submersed in 70% ethanol for 1 min followed by addition of 10% sodium hypochlorite containing 0.2% Tween-20 with vigorous shaking for 20 min. Seeds were rinsed five times with a large excess of sterile water (2).

### **Inoculation and Planting of Sterilized Seeds**

Sterilized bean and wheat seeds were inoculated with either the wildtype *P. putida* culture or J-1 culture by adding the seeds to a flask of the culture for 3-5 minutes. Each culture had been grown to stationary phase (24-30 hrs) in LB media with nalidixic acid. Inoculated seeds were planted in Magenta boxes that had been filled with a mixture of 300 mL vermiculite and 150 mL water and autoclaved two times, 24 hrs apart. Five wheat seeds were planted for each wheat box and three bean seeds for each of these boxes. For controls, uninoculated but sterilized bean and wheat seeds were also planted. The Magenta boxes were incubated for plant growth at 26°C under light.

### **Preparation of Root Washes**

At 10 days and 16 days after planting, root washes were prepared to release bacterial cells. Excess vermiculite was removed from the root by gentle shaking. A single root was added to 10 mL of sterile water and vortexed for 10 seconds. This root wash was serially diluted and a 20 microliter aliquot of each dilution was plated on King medium B (KB) agar (9). Plates were incubated at 26°C for 3 days before cell counts were made.

### **Quantification of Bacterial Release from Plant Roots**

Plates were observed for growth and the number of colonies recorded. From the data collected, outliers were determined through the Dixon test and those points were replaced with the next closest value (winsorized) (11). A mean and standard error were then calculated for each data set.

### **Catalase Test on Cells Recovered from Root Washes**

A sample of wildtype and J-1 mutant cells that were recovered from bean and wheat root washes through plating on KB media were restreaked onto new KB agar plates for catalase testing. A cluster of

colonies from these plates was treated with a 1:10 dilution of hydrogen peroxide (0.88mM) and sterile water. The appearance of oxygen bubbles from the treated cells indicated a positive result.

## RESULTS

The data obtained for bean root colonization by the catalase A-deficient mutant (J-1) as compared to the wildtype strain are presented in Table 1 as means and standard errors. The value *n* refers to the number of roots that were washed separately.

At Day 10 after planting, level of bean root colonization by the wildtype and J-1 mutant was similar. At Day 16, J-1 mutant colonization exceeded wildtype colonization. For both the wildtype and catalase A-deficient mutant, fewer bacteria were recovered from the bean roots at Day 16 as compared to Day 10. Root wash dilutions for the uninoculated but sterilized bean seeds showed no bacterial growth.

**TABLE 1. Bean root colonization by catalase A-deficient mutant(J-1) and *P. putida* wildtype**

TIME POINT	WILDTYPE 10 <sup>6</sup> CFU/g root	J-1 MUTANT 10 <sup>6</sup> CFU/g root
Day 10	11 ± 3.7 (n=3)	14 ± 5.2 (n=3)
Day 16	3.1 ± 0.6 (n=5)	11 ± 1.8 (n=6)

Sterilized seeds were inoculated at planting with cells grown to stationary phase (24-30 hrs). Each plant root was washed and a dilution series performed at Day 10 or 16 after planting. The dilution series were plated on King medium B agar with nalidixic acid and incubated at 26°C for 3 days before cell counts were made. The values presented are means ± standard error.

Table 2 shows the data obtained for wheat root colonization by the J-1 mutant as compared to the wildtype. As with bean, a higher number of J-1 mutant cells were recovered from wheat roots at Day 16 as compared to wildtype cells. At Day 22, more wildtype cells were recovered, but only one wildtype root wash dilution series was counted for this time period. Similar numbers of J-1 mutant cells were recovered at Day

16 and Day 22. The one wildtype root wash dilution analyzed for Day 22 showed a greater number of cells than were recovered at Day 16.

**TABLE 2. Wheat root colonization by catalase A-deficient mutant (J-1) and *P. putida* wildtype**

TIME POINT	WILDTYPE 10 <sup>6</sup> CFU/g root	J-1 MUTANT 10 <sup>6</sup> CFU/g root
Day 16	2.8 ± 1.0 (n=4)	4.3 ± 0.5 (n=3)
Day 22	10 (n=1)	4.0 ± 0.6 (n=3)

Sterilized seeds were inoculated at planting with cells grown to stationary phase (24-30 hrs). Each plant root was washed and a dilution series performed at Day 10 or 16 after planting. The dilution series were plated on King medium B agar with nalidixic acid and incubated at 26°C for 3 days before cell counts were made. The values presented are means ± standard error.

Samples of wildtype cells from the dilution series plates streaked onto new KB plates, from each time point and from both bean and wheat roots, showed a release of oxygen bubbles when treated with hydrogen peroxide. Each J-1 mutant cell evolved no oxygen bubbles.

### DISCUSSION

A greater number of J-1 mutant cells were recovered from bean roots at Day 10 and Day 16 after planting and from wheat roots at Day 16 as a compared to wildtype cells (Table 1, Table 2). The only point where the wildtype colonized at higher levels than the catalase A-deficient mutant was Day 22 of the wheat root colonization. For this point, only one root was used to determine the number of wildtype cells released, and the value is therefore not as accurate as the others listed which are means figured from three or more roots. The data collected could indicate that the catalase A-deficient mutant was generally as effective at colonizing the bean and wheat roots as the wildtype. However, there are also various potential sources of error and alternate explanations.

The potential sources of error begin with seed inoculation. By placing the sterilized seeds in a flask



of culture that had been grown to stationary phase for inoculation, we have no way of knowing how many of these cells actually adhered to each seed. Therefore, the precise inoculum value for the wildtype and J-1 mutant bean and wheat seeds cannot be known. It is possible, for example, that the initial inoculum of one isolate was more concentrated than another, and this in turn would affect the number of cells recovered through root washes. To obtain a figure for the number of cells included in the initial inoculum, a dilution series could have been carried out for washes from the seed at the time of planting.

The method for preparing root washes could also introduce variability in the number of bacteria removed from the root. For example, the manner in which the root system was removed from the Magenta box could affect the recovery of cells colonizing the root. Also, the vermiculite that remained adhered to the root may have interfered with bacterial release from the root.

Although a catalase test was used on the cells recovered, because it was performed on a mass of colonies from the plated dilutions and not individual colonies, it can not be concluded that all of the J-1 mutant organisms collected were still catalase A-deficient.

It is important to consider the method by which the J-1 mutant was formed in drawing conclusions from the data obtained. The J-1 mutant was developed through chemical mutagenesis with ethyl methanesulfonate (EMS). The mutant strain may have various unknown mutations in addition to its lack of catalase A that could affect its growth on the root. A catalase A-deficient mutant developed through site-directed mutagenesis would eliminate this unknown dimension.

The results obtained do not confirm my hypothesis that catalase A-deficient mutant cells would be less effective colonizers than wildtype *P. putida* cells that have catalase activity. Two alternate conclusions may be drawn from these results. First, effective root colonization by *P. putida* does not require catalase activity: Catalase A mutants can colonize at levels comparable to the wildtype. Second, it is possible that cells recovered from the root were not catalase A-deficient mutants but had reverted at the plant root to express catalase A. It is also possible that catalase A-deficient cells began to express catalase isozymes B

and C at the plant root but this possibility was not tested.

Additional studies using more plant roots, a catalase A mutant formed by site-directed mutagenesis, and a catalase test of individual cells recovered from the J-1 inoculated root would help to determine the actual role of catalase A in root colonization by *P. putida*.

### III. ROOT COLONIZATION BY A CATALASE C-DEFICIENT MUTANT

#### INTRODUCTION

The role of catalase C in root colonization by *P. putida* was studied using surface sterilized bean and wheat seeds inoculated with either the catalase C-deficient mutant or the wildtype strain and planted in sterile vermiculite. The catalase C mutant was formed through site-directed chromosomal mutagenesis. A cassette with the luciferase genes (*lux AB*) and a kanomycin resistant gene was ligated into the catalase C gene of *P. putida*. The expression of the *lux AB* is regulated by the catalase C gene promoter. Luciferase catalyzes a reaction that produces a blue-green light (10). This luciferase activity can be measured and indicates transcription from the catalase C gene promoter in the mutant organism.

#### MATERIAL AND METHODS

*P. putida* *nal<sup>R</sup>* isolate Corvallis and a catalase C-deficient mutant, formed through site-directed chromosomal mutagenesis, were stored at -80°C in 15% glycerol (50 µg/mL). For culture, cells were transferred into liquid LB media and grown to stationary phase (24-30 hrs).

Procedures for the sterilization, inoculation and planting of seeds were identical to those described in Section II (pp. 4-5). Root washes were prepared as described in Section II (pg 5). Two different experiments were conducted. For Experiment 1, root washes were prepared at 10 days and 16 days after

planting. For Experiment 2, root washes were prepared at 7 days and 14 days after planting.

Procedures for the quantification of bacterial release from roots and the catalase test were identical to those described in Section II (pg 5 and 6).

## RESULTS

The data obtained for bean root colonization by the catalase C mutant and wildtype strain are presented in Table 3 as means and standard errors. The value n refers to the number of roots that were washed separately. Dilution series of uninoculated but sterilized seeds showed no bacterial growth.

Bean root colonization in Experiments 1 and 2 revealed similar trends. At the first time point of each experiment (Day 10 for Experiment 1 and Day 7 for Experiment 2), more wildtype cells were recovered from bean roots than catalase C-deficient cells. At the second time point (Day 16 for Experiment 1 and Day 14 for Experiment 2), a higher number of catalase C-deficient cells were recovered as compared to wildtype cells. For both the wildtype and mutant cells, both experiments generally showed a decrease in the number of cells recovered at the second time point as compared to the first. The exception to this trend is the second time point of Experiment 2 for the catalase C-deficient cells.

**TABLE 3. Bean root colonization by catalase C mutant and *P. putida* wildtype**

TIME POINT	WILDTYPE 10 <sup>6</sup> CFU/g root	CATALASE C MUTANT 10 <sup>6</sup> CFU/g root
Experiment 1, Day 10	11 ± 3.7 (n=3)	5.0 ± 1.6 (n=3)
Experiment 1, Day 16	3.1 ± 0.6 (n=5)	4.3 ± 0.8 (n=3)
Experiment 2, Day 7	18 ± 0.3 (n=4)	7.0 ± 0.5 (n=4)
Experiment 2, Day 14	10 ± 5.8 (n=4)	19 ± 9.3 (n=4)

Sterilized seeds were inoculated at planting with cells grown to stationary phase (24-30 hrs). Each plant root was washed and a dilution series performed at the time point indicated. The dilution series were plated on King medium B agar with nalidixic acid and incubated at 26°C for 3 days before cell counts were made. The values presented are means ± standard error.



Wheat root colonization is shown in Table 4. No data for wildtype cells from the first time point of Experiment 1 were available to compare with the number of mutant cells recovered. In the first time point of Experiment 2 (Day 7), a higher number of wildtype cells were recovered from wheat roots as compared to mutant cells. At the second time point of both experiments (Day 16 for Experiment 1 and Day 14 for Experiment 2), a higher number of catalase C-deficient cells were recovered. This trend is similar to that observed in bean root colonization by the catalase C-deficient mutant

**TABLE 4. Wheat root colonization by catalase C mutant and *P. putida* wildtype**

TIME POINT	WILDTYPE 10 <sup>6</sup> CFU/g root	CATALASE C MUTANT 10 <sup>6</sup> CFU/g root
Experiment 1, Day 10	No value	4.3 ± 0.7 (n=5)
Experiment 1, Day 16	2.8 ± 1.0 (n=4)	3.7 ± 0.3 (n=3)
Experiment 2, Day 7	16 ± 3.4 (n=3)	5.5 ± 0.4 (n=3)
Experiment 2, Day 14	11 ± 4.1 (n=4)	12 ± 2.8 (n=4)

Sterilized seeds were inoculated at planting with cells grown to stationary phase (24-30 hrs). Each plant root was washed and a dilution series performed at the time point indicated. The dilution series were plated on King medium B agar with nalidixic acid and incubated at 26°C for 3 days before cell counts were made. The values presented are means ± standard error.

For experiment 1, a sample of wildtype and catalase C-deficient cells from the Day 10 and Day 16 dilution plates was streaked onto new KB agar plates. A cluster of the new colonies was catalase-tested with a 1:10 dilution (0.88 mM) of hydrogen peroxide in sterile water. Oxygen bubbles released from the treated cells indicated a positive test. Both the wildtype cells and catalase C mutant cells test were catalase positive.



## DISCUSSION

For the two experiments conducted on both bean and wheat roots, a greater number wildtype cells were generally recovered at the first time point and a higher number of catalase C-deficient cells were recovered at the second time point. The first time point of Experiment 1 for wheat root colonization can not be used to compare wildtype and mutant colonization levels because no data were available for wildtype cells recovered from the roots.

In the bean root colonization experiments, both organisms showed a general decrease in the number of cells recovered at the second time point as compared to the first. The wheat root colonization experiments do not show as consistent of a pattern. For example, Experiment 1 showed a decrease in catalase C-deficient cells recovered at the second time point, but Experiment 2 showed an increase in the number of mutant cells recovered at the second time point.

The catalase test of a cluster of colonies from Experiment 1 (Day 10 and Day 16) dilution plates was positive for both wildtype and catalase C-deficient cells. This result is expected for the mutant cells, because the site-directed mutagenesis only eliminates catalase C activity. The mutated organism still has the catalase A and B isozymes active. Because of this, a catalase test is not a very useful way to determine if the cells recovered from the roots were still catalase C-deficient cells. Quantifying luciferase activity of the presumed catalase C-deficient cells would have been an effective way of verifying that the cells were still catalase C-deficient.

These findings indicate that the presence of catalase isozyme C in *P. putida*, may not be necessary for effective root colonization perhaps because the catalase C-deficient mutants still have catalase activity from the A and B isozymes. As mentioned, these experiments showed greater colonization by the mutant cells as compared to the wildtype cells at the second time point.

#### IV. COLONIZATION BY A SUPEROXIDE DISMUTASE-DEFICIENT MUTANT

##### INTRODUCTION

Superoxide dismutase (SOD) is an enzyme that dismutates superoxide anion to hydrogen peroxide and molecular oxygen. To examine the role of SOD, colonization by wildtype *P. putida* or a SOD mutant on bean and wheat roots was compared.

*P. putida* has two isozymes of SOD, the FeSOD and MnSOD. The MnSOD is only induced in iron-deficient conditions. The SOD mutant used in these experiments lacks the FeSOD isozyme because of site-directed chromosomal mutagenesis. A cassette with the luciferase genes (*lux AB*) and a kanomycin resistant gene was ligated into the FeSOD gene of *P. putida*. The expression of the luciferase genes is regulated by the FeSOD gene promoter. Luciferase catalyzes a reaction that produces a blue-green light (10). This luciferase activity can be measured and indicates transcription from the promoter of the ligated FeSOD gene.

##### MATERIALS AND METHODS

*P. putida* *nal<sup>R</sup>* isolate Corvallis and a SOD mutant, formed through site-directed chromosomal mutagenesis, were stored at -80°C in 15% glycerol (50 µg/mL). For culture, cells were transferred into liquid LB media and grown to stationary phase (24-30 hrs).

Procedures for surface sterilization of bean and wheat seeds and inoculation and planting of seeds were identical to those described in Section II (pp.4-5). The control boxes for Experiment 2 were different than the controls of Experiment 1. In Experiment 1, uninoculated but sterilized bean and wheat seeds were planted. For Experiment 2, 100 microliters of culture was inoculated into the center of the vermiculite in two magenta boxes (per organism). No seeds were planted in these control boxes.

For experiment 1, root washes were prepared on Day 7 and Day 14 after planting as described in

Section II (pg 5). For Experiment 2, the same procedure was used at Day 5 and Day 14 with the following differences. Eight plant roots were used per root wash as compared to one plant root per root wash in Experiment 1. Wheat plants were not available for Experiment 2. The bean root washes were filtered through 6 layers of cheesecloth to remove vermiculite particles. Serial dilutions of the root washes were plated on both King medium B and LB agar for bacterial growth. For the control boxes, a ~2 g sample of the inoculated but unseeded vermiculite was collected for each organism and mixed with 20 mL of sterile water. Serial dilutions of these control washes were prepared and plated on KB and LB agar plates.

#### **Preparation of Extracts for Nondenaturing Gel Electrophoresis**

Cells were obtained from the root washes by centrifugation and resuspension in 3 mL of 50mM potassium phosphate, pH 7.0. Each cell suspension on ice was exposed to sonication for 20 second bursts. The debris was removed by centrifugation at 15,000 x g for 10 min and the supernatant was stored at -70°C. Isozyme analyses for superoxide dismutase were performed using nondenaturing gel electrophoresis as described by Katsuwon and Anderson (1989).

Procedures used for the quantification of bacterial release from plant roots were identical to those described in Section II (pg 5).

#### **Investigation of SOD Mutant Colony Size**

Colony size of the SOD mutants was observed by culturing cells in liquid LB media until stationary phase (24-30 hrs) and streaking onto KB and LB agar plates. The plates were incubated for growth at 26°C for 3 days under light.

#### **Quantification of Luciferase Activity in SOD Mutant**

Luciferase activity of the SOD mutant cells recovered from bean roots of Experiment 2, Day 14 and from the vermiculite inoculated with mutant culture from Experiment 2, Day 14 was monitored with a luminometer. The substrate solution used consisted of 10 microliters of n-decanol and 9990 microliters of ethanol. Ten microliters of the substrate was added to 990 microliters of a root wash sample and luciferase

activity was recorded in relative lux units per microliter for a 10 second reading.

## ***RESULTS***

### **Root Colonization Data**

The data obtained for bean and root colonization by the SOD mutant are presented in Table 5. The SOD mutant colonies at Day 7 of Experiment 1 were much smaller in size than the wildtype colonies. The same difference in colony size was observed in Experiment 2. Here, a mix of small and large SOD mutant colonies was observed at Day 5. The colonies were differentially counted and recorded in Table 5. Mixed colony size was observed on both KB agar and LB agar at Day 5. At Day 14 of both experiments, the colonies from each strain were large and thus more similar in appearance.

Wheat root colonization by the SOD mutant and the wildtype is shown in Table 6. The same difference in colony size of SOD mutants observed in cells recovered from bean roots was found in cells recovered from wheat roots of Experiment 1. No wheat plants were available for testing in Experiment 2. At Day 7 and Day 14, higher numbers of wildtype cells than the SOD mutant were recovered from the wheat roots.



**TABLE 5. Bean root colonization by SOD mutant and *P. putida* wildtype**

TIME POINT	WILDTYPE 10 <sup>6</sup> CFU/g root	SOD MUTANT 10 <sup>6</sup> CFU/g root
Experiment 1, Day 7	20 ± 0.3 (n=4)	Large: None Small: 8.6 ± 2.3 (n=4)
Experiment 1, Day 14	10 ± 5.8 (n=4)	Large: 35 ± 8.5 (n=3) Small: None
Experiment 2, Day 5 KB media	25 ± 6.4 (n=3)*	Large: 20 ± 4.1 (n=3)* Small: 77 ± 24 (n=3)*
LB media	15 ± 4.1 (n=3)*	Large: 15 ± 5.0 (n=3)* Small: 42 ± 20 (n=3)*
Experiment 2, Day 14 KB media	100 ± 35 (n=2)*	Large: 75 ± 18 (n=2)* Small: None
LB media	250 ± 106 (n=2)*	Large: 65 ± 25 (n=2)* Small: None

\*For experiment 2, eight roots were used in one root wash. Therefore, *n* represents the number of root washes each with eight roots. Experiment 1 root washes contained only one root.

Sterilized seeds were inoculated at planting with cells grown to stationary phase (24-30 hrs). Dilution series were prepared for root washes and plated on King medium B agar for Expt. 1 and KB and LB agar for Expt. 2 (All plates contained 50 µg/mL nalidixic acid). Plates were incubated at 26°C for 3 days before cell counts were made. The values presented are means ± standard error. For experiment 1, at Day 7 the SOD colonies were much smaller in size than the wildtype colonies. At Day 14 of experiment 1, the colonies from each isolate were more similar in appearance. The same differences were observed in the second experiment, and counts were made for each size of SOD mutant colony. The values presented are means ± standard error.

**TABLE 6. Wheat root colonization by SOD mutant and *P. putida* wildtype**

TIME POINT	WILDTYPE 10 <sup>6</sup> CFU/g root	SOD MUTANT 10 <sup>6</sup> CFU/g root
Experiment 1, Day 7	16 ± 3.4 (n=3)	3.3 ± 0.9 (n=4)
Experiment 1, Day 14	11 ± 4.1 (n=4)	5.3 ± 1.4 (n=4)

Sterilized seeds were inoculated at planting with cells grown to stationary phase (24-30 hrs). Each plant root was washed and a dilution series prepared at day 7 and day 14. Dilution series were plated on King medium B agar with nalidixic acid. Plates were incubated at 26°C for 3 days before cell counts were made. The values presented are means ± standard error. At day 7 the SOD colonies were much smaller in size than the wildtype colonies. At day 14, the colonies from each isolate were more similar in appearance.

### Colony Size of SOD Mutant Cells

SOD mutant colonies that grew from stationary phase cells (24-30 hr culture) raised from freezer stocks were all small in size and the wildtype colonies were all large in size. The results shown in Table 7 are for cells inoculated into the vermiculite of two Magenta boxes (per organism) without seeds. Values presented are means and standard errors. The SOD mutant colonies were a mix of small and large colonies, with more small colonies observed at Day 5 as compared to Day 14. The two colony types were not differentiated in counting the cells. Growth of the SOD mutant cells and wildtype cells on KB and LB media was comparable. For both media types, a greater number of wildtype cells was recovered from the control Magenta boxes at Day 5 and Day 14 as compared to SOD mutant cells recovered. In addition, the number of cells recovered for each organism was slightly greater at the second time point (Day 14) as compared to the first (Day 5).

**TABLE 7. Growth of wildtype and SOD mutant from vermiculite control samples**

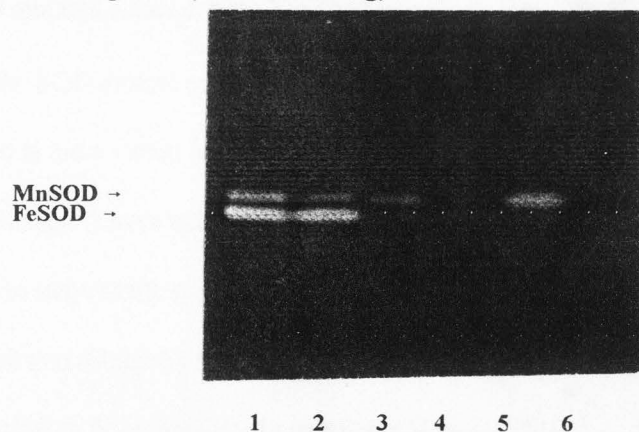
TIME POINT	WILDTYPE CONTROL 10 <sup>6</sup> CFU/g root	SOD MUTANT CONTROL 10 <sup>6</sup> CFU/g root
Experiment 2, Day 5 KB media	10 ± 3.5 (n=2)	Mix of large and small colonies: 2.3 ± 1.8 (n=2)
LB media	10 ± 0.0 (n=2)	Mix of large and small colonies: 2.1 ± 1.3 (n=2)
Experiment 2, Day 14 KB media	18 ± 5.3 (n=2)	Mix of large and small colonies*: 5.0 ± 0.0 (n=2)
LB media	20 ± 11 (n=2)	Mix of large and small colonies*: 7.2 ± 5.2 (n=2)

Sterilized vermiculite in Magenta boxes was inoculated with 100 microliters of culture for controls. A ~2 g sample of the inoculate vermiculite was mixed with 20 mL of sterile water at day 5 and day 14. A dilution series was prepared and plated on King medium B and LB media with nalidixic acid. Plates were incubated for growth at 26°C for 3 days before counts were made. \*The cell numbers reported were a mix of large and small cells. At day 14 there were fewer small colonies than at day 5.

### SOD Isozyme Analysis

The nondenaturing gel electrophoresis of bean root wash samples (60 microliters per lane) from Experiment 2 was not sensitive enough to detect any SOD activity from the wildtype or SOD mutant cells. Gel electrophoresis of extracts from wildtype stationary phase cells grown from freezer stocks with  $\text{MnCl}_2$  showed two bands corresponding to the FeSOD and MnSOD isozymes (Figure 1, Lanes 1 and 2). The MnSOD band was more intense in the 48 hr culture. Samples of SOD mutant small and large colonies cultured from the Experiment 2, Day 5 bean root wash dilutions were added to liquid LB media with no  $\text{MnCl}_2$  and grown to stationary phase. Extracts were prepared from these cultures. Gel electrophoresis of the SOD mutant large colony extracts showed a strong MnSOD band (Figure 1, Lanes 3 and 5). Extracts from SOD mutant small colonies did not show MnSOD activity (Figure 1, Lanes 4 and 6).

**FIG. 1. Nondenaturing gel electrophoresis of *P. putida* wildtype and SOD mutant samples. (SOD staining)**



Lane 1: Wildtype cells from freezer stocks (with  $\text{MnCl}_2$ ), Day 2 growth  
Lane 2: Wildtype cells from freezer stocks (with  $\text{MnCl}_2$ ), Day 1 growth  
Lane 3: SOD mutant large colonies grown in liquid LB media  
Lane 4: SOD mutant small colonies grown in liquid LB media  
Lane 5: SOD mutant large colonies grown in liquid KB media  
Lane 6: SOD mutant small colonies grown in liquid KB media



### Luciferase Expression of SOD Mutants

Luciferase activity was expressed in the SOD mutant cells recovered from the bean root washes and SOD mutant cells from vermiculite of Experiment 2, Day 14. The data obtained (Table 8) show a large range of activity.

**TABLE 8. Luciferase activity of SOD mutants recovered from control vermiculite samples and bean roots (Experiment 2, Day 14)**

<b>SOURCE OF SOD MUTANT CELLS ANALYZED</b>	<b>RLU's (lux unit/mL sample)</b>
SOD Mutant Cells from Vermiculite Control	27,889
SOD Mutant Cells from Bean Root Sample 1	76,048
SOD Mutant Cells from Bean Root Sample 2	383,400

### *DISCUSSION*

FeSOD mutants colonize both bean and wheat roots. Wildtype cells colonized wheat roots at higher levels than did the SOD mutant cells for Day 7 and Day 14 after planting. The bacterial counts from bean root colonization is more varied and difficult to interpret because of the appearance of two different sizes of SOD mutant colonies. I have called these sizes large and small colonies. The size increased from small in the early assays to large as the seedlings matured. The use of LB or KB agar media did not affect the detection of these two colony types.

Samples taken from inoculated vermiculite alone showed the same trend in altered colony size. For each media at day 5 and day 14, more wildtype cells were recovered from the inoculated vermiculite.

Unfortunately, gel electrophoresis carried out on wildtype and SOD mutant cells directly recovered from the bean root washes of Experiment 2 was not sensitive enough to detect SOD activity. A more concentrated sample or more plant roots per wash (at least greater than 8, the number used in Experiment 2) may permit this analysis. SOD mutant cells recovered from the vermiculite controls and from bean root



washes expressed luciferase activity. This verifies that the cells recovered are the SOD mutant cells and indicates that the promoter of the FeSOD gene is active.

The small and large colony types of the SOD mutant may be related to the activity of the two SOD isozymes, FeSOD and MnSOD. In wildtype cells, MnSOD is only induced in iron-deficient conditions. The SOD mutant used in these experiments lacks the FeSOD isozyme and extracts from the small cell type show little activity on electrophoresis. It is possible that the large SOD mutant colonies are large because the MnSOD isozyme has been induced, while the small colonies are not growing as quickly because they lack both isozymes of SOD. Gel electrophoresis of SOD mutant small and large colonies cultured on LB agar media from root wash dilutions confirm that the SOD mutant large colonies have MnSOD activity but the small colonies do not. Because the large SOD mutant colonies are observed as cultures mature, one could suggest that starvation is inducing the MnSOD activity.

In conclusion, SOD mutant cells recovered from bean and wheat root washes showed small colony size at early time points and large colony size at later time points. The SOD mutant cells recovered from bean roots exhibited luciferase activity and colonized bean and wheat roots at levels comparable to the wildtype. It is possible that the root environment and/or starvation induces the MnSOD isozyme after a certain amount of time on the root. The induction of this isozyme may explain the larger SOD mutant colonies observed at the later time points of both experiments.

## V. OVERALL DISCUSSION AND CONCLUSION

In comparing root colonization by the wildtype *P. putida* organism with colonization by a catalase A-deficient mutant, a catalase C-deficient mutant, and a SOD-deficient mutant, the mutant organisms were found to colonize bean and wheat roots at levels generally comparable to that of the wildtype organism. I hypothesized that catalase or SOD-deficient organisms would be less effective root colonizers because of their inability to break down the toxic activated oxygen species produced by surface enzymes of the plant

root. The data collected for root colonization by each mutant do not affirm this hypothesis.

This study of catalase and superoxide dismutase isozymes and their importance in bean and wheat root colonization by *P. putida* is a lesson in microbial adaptability. Although the mutants studied were deficient in one isozyme, the presence of other isozymes may allow the bacteria to effectively adapt to the conditions at the plant root. For the catalase A-deficient mutant, the catalase isozymes B and C may have been induced and sufficient to protect the cells. Similarly, disruption of the catalase C gene does not appear to inhibit root colonization by the mutant. The FeSOD mutant cells appear able to induce the second isozyme, MnSOD, under certain conditions. Certainly, *P. putida* appears to be well-equipped to respond to various environmental pressures.

In conducting these experiments I have not only gained an appreciation for the adaptability of *P. putida*, but have learned many research techniques that are useful in the study of microorganisms. I have a better understanding of how best to plan experiments to address questions and test hypotheses. Much of this knowledge was illuminated *after* my research was complete! In retrospect, I can appreciate the importance of controls in supporting the integrity of your study and the importance of consistently and accurately determining the nature of the organism being analyzed. This was especially true in the study of mutants because of the microorganism's ability to adapt to certain conditions and induce enzymatic activity.

The interaction of *P. putida* cells with plant roots is complex and various types of experiments and research techniques must be used to address the importance of catalase and superoxide dismutase isozymes in root colonization. It is my hope that the undergraduate research I have done with this organism will in some way assist others in answering the many questions that remain.

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