# MONITORING FOR GENETICALLY ENGINEERED PSEUDOMONAS SPECIES IN MONTEREY COUNTY

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BIOLOGICAL CONTROL SERVICES PROGRAM

State of California
Department of Food and Agriculture
Pest Management, Environmental Protection
and Worker Safety
Environmental Monitoring and Pest Management
1220 N Street, Sacramento, CA 95814

EH 87-03

# MONITORING FOR GENETICALLY ENGINEERED

# PSEUDOMONAS SPECIES IN MONTEREY COUNTY

by

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## ABSTRACT

A field monitoring study was conducted to determine if genetically altered Pseudomonas fluorescens or P. syringae had been applied to sites in Monterey County. A series of diagnostic tests for antibiotic resistance, fluorescence ability, oxidase and arginine dihydrolase activities, hypersensitivity reaction and ice nucleation ability were conducted to screen bacteria isolated from field and control samples. No bacteria were detected from field samples which matched the expected test profiles of genetically altered bacterial products. In contrast, bacteria were consistently isolated from positive control samples with the expected characteristics of genetically altered bacteria.

**ACKNOWLEDGMENTS**

Thanks to Richard W. Nutter, Monterey County Agricultural Commissioner and his staff for their assistance in planning and carrying out the study plan.

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## INTRODUCTION

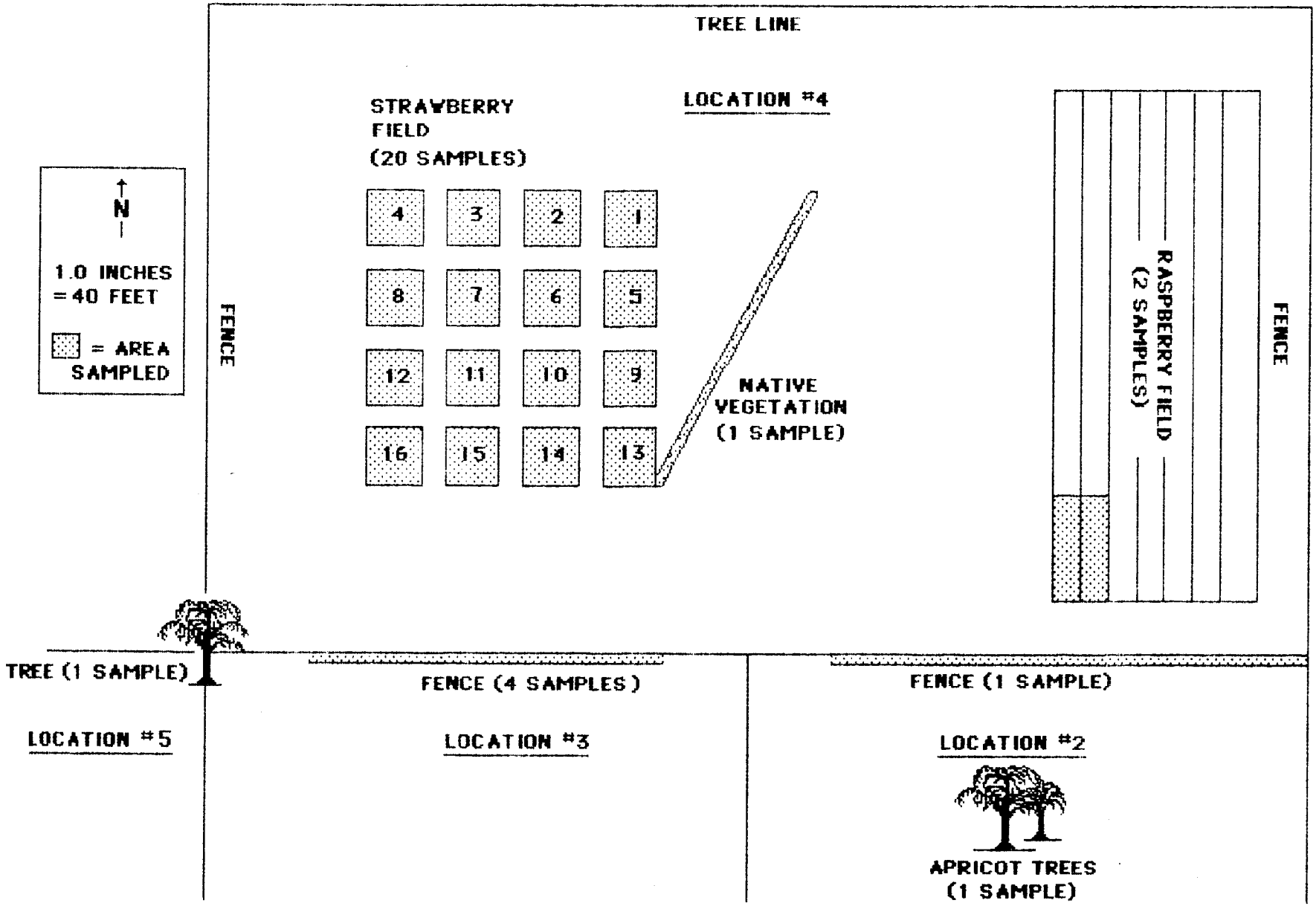
Naturally occurring ice nucleation active (INA) epiphytic bacteria have been shown to be important catalysts of frost formation and crop injury (4). This ability to initiate ice formation is a genetically determined property. Through gene deletion, wild-type INA bacteria can be altered so that they no longer have the ability to initiate ice formation. These altered bacteria are referred to as INA- or "ice minus" bacteria. Several such products are presently being developed which use gene deletion to engineer strains of Pseudomonas species. When applied to crops these genetically engineered Pseudomonas strains are intended to provide an alternative form of frost control (3). Approval for the environmental release of these products was granted by state and federal agencies responsible for the regulation of microbial pesticides (2). However, an experimental release was never officially conducted. At the request of the Monterey County Health Department, the California Department of Food and Agriculture (CDFA) initiated a study to determine if genetically altered Pseudomonas fluorescens or P. syringae had been applied to sites in Monterey County without proper notification of state and federal agencies.

## MATERIALS AND METHODS

### Sampling Locations

Thirty-two samples of vegetation were collected at random from five properties in Monterey County (see Figure 1) on March 26, 1986. Simultaneously, eight positive control samples were collected from strawberry plants located in an Advanced Genetics Sciences, Inc. (AGS) greenhouse in Oakland, California. Each sample contained 24 leaves or flowers.

FIGURE 1. CALIFORNIA DEPARTMENT OF FOOD AND AGRICULTURE ENVIRONMENTAL MONITORING BRANCH ICE MINUS SAMPLING LOCATIONS, MARCH 26, 1986. NEGATIVE CONTROL SITE (LOCATION #1) WAS APPROXIMATELY ONE MILE SOUTH OF THIS AREA.



The original sampling plan specified that a total of 30 samples would be collected from 4 properties in Monterey County: 20 samples from a strawberry field which AGS originally intended to use for the experimental release of genetically altered bacteria; 4 samples collected from each of two properties adjacent to this strawberry field; and two control samples from one additional property. After discussions with the Agricultural Commissioner's staff, changes in this plan were made which included the selection of an additional sampling location.

Location #1 was located approximately one mile from the strawberry field, and used as a negative control. Two replicate leaf samples were collected at this property from a variety of species. Locations #2 and #3 were located adjacent and south of the strawberry field property. Two leaf samples were collected at location #2. One sample included leaves from a variety of species found along the back fence which separated the property and the strawberry field. The second sample consisted of leaves collected from two apricot trees in the front yard of this property. At location #3, four replicate leaf samples were collected from a variety of species found along the back fence which separated the property and the strawberry field.

Location #4 is the property on which the strawberry field was located. The field was approximately 82 x 82 feet and consisted of 16 plots, each approximately 16 x 16 feet. Leaf samples were collected at random, one from each plot. Four flower samples were collected, each consisting of flowers selected at random from four plots. A small raspberry field was also located on this property. Two leaf samples were collected at random from a 16 x 30 foot area in the southwest corner of the field. This was the corner closest to the adjacent properties and the

strawberry field. An area of natural vegetation adjacent to the strawberry field was also sampled by collecting leaves at random along a diagonal transect through this area.

Location #5 was sampled at the request of the Agricultural Commissioner. One leaf sample was collected from a tree in the northeast corner of this property. This tree overhangs into Location #4, and was sampled from that side.

Plants for positive control samples were prepared by AGS (location #6). Six leaf and two flower positive control samples were collected from strawberry plants located in an AGS greenhouse. These strawberry plants had been sprayed two days earlier with a suspension of $3.7 \times 10^8$ and $7.8 \times 10^8$ cells per ml, respectively, of genetically altered strains of P. syringae or P. fluorescens, either alone (four samples) or in combination (four samples).

**Sampling Methods**

Individual leaves and flowers were cut from stems with hand clippers and placed in ziplock plastic bags. Gloves and shoe covers were changed for each property, and the clippers were washed with isopropyl alcohol after each sample was collected. Accompanying each sample was a chain of custody form on which all pertinent sampling data as well as all persons handling the sample were recorded (see Appendix I).

After collection, all samples were immediately cooled with wet ice, and transported to Sacramento. All samples were kept refrigerated in a locked room until they were given to the CDFA Analysis and Identification Branch the next day. Positive control samples were submitted "blind" to the laboratory along with the other samples.

## Isolation and Characterization of Bacteria

Each sample was added to a sterile 250 ml flask containing 125 ml of sterile buffer before being placed in a shaker for two hours to remove bacteria from plant surfaces by washing action. After shaking, 0.1 ml aliquots of wash buffer were removed from each sample and placed on 10 replicate plates for each of two selective media.

Both selective media consisted of Kings B medium modified by the addition of cycloheximide, and either rifampicin or nalidixic acid. The Kings B medium is selective for Pseudomonas species, while the cycloheximide inhibits fungal growth. Rifampicin and nalidixic acid are antibiotics to which AGS products have resistance. Culture plates were spun on a turntable and inoculum spread with a sterile glass rod. Plates were sealed with parafilm, inverted and incubated at room temperature (approximately 21-24°C). After incubation for 48 to 72 hours, growth on modified King's B media was evaluated. As a control, amended King's B media was also inoculated with isolates of genetically altered P. fluorescens or P. syringae provided by AGS.

Genetically altered P. fluorescens and P. syringae produced by AGS have distinctive antibiotic resistance due to selection by AGS from wild type bacteria, as well as specific properties possessed by each species. These distinguishing characteristics were the basis for a series of tests conducted to screen bacterial isolates from field and control samples. Bacterial isolates were tested to determine if they fit the expected profile for AGS products (see Table 1 for expected profile). Bacterial isolates which did not match this expected profile were eliminated from further consideration as being the genetically engineered bacteria produced by AGS and no further testing was required.

Table 1. Expected diagnostic test profile of genetically engineered Pseudomonas species supplied by AGS.

| | P. syringae | P. fluorescens |
|---|---|---|
| | Nalidixic Acid resistant | Rifampicin resistant |
| Fluorescence | + | + |
| Oxidase | - | + |
| Arginine dihydrolase | - | + |
| Hypersensitivity | + | - |
| Ice nucleation activity | - | - |

Because both P. syringae and P. fluorescens are fluorescent pseudomonads, all bacterial colonies growing on modified King's B media were examined under long wavelength ultraviolet light. If fluorescent colonies were present, one representative fluorescent colony per plate was selected for isolation and further characterization.

Genetically altered Pseudomonas strains produced by AGS have distinctive antibiotic resistance profiles. P. syringae and P. fluorescens strains are resistant to nalidixic acid and rifampicin, respectively. To determine whether fluorescent bacteria from field and control samples matched the expected profile for the genetically engineered bacteria, isolates from the selective media were evaluated using additional diagnostic tests which included oxidase, arginine dihydrolase, and hypersensitive reaction tests. In the oxidase test, bacteria are streaked on filter paper and spotted with oxidase reagent. P. fluorescens gives a positive blue color response within one minute, while P. syringae gives a negative response. The arginine dihydrolase test involves inoculation of a tube containing arginine media with test bacteria. After covering the media with mineral oil and incubating for 24 hours, media will turn pink if the response is positive. For this test, P. fluorescens and P. syringae give positive and negative responses, respectively. In testing for hypersensitivity reaction (HR), a bacterial suspension is injected into a tobacco leaf. In a positive reaction, leaf necrosis appears within 24 hours of inoculation. P. syringae is expected to be HR positive, while P. fluorescens is expected to be HR negative.

Following diagnostic tests for species identification, isolates were further tested for ice nucleation activity. In this test for each isolate 0.1 ml of a $10^8$ bacterial suspension was added to a test tube containing 10 ml of water

pre-cooled to -5°C. Bacteria capable of catalyzing ice formation at -5°C were considered ice nucleation active.

## RESULTS

No fluorescent colonies were isolated from field samples on Kings B media amended with rifampicin (see Table 2). This was evidence that no genetically engineered P. fluorescens was present in the field samples. Twelve field samples yielded a small number of fluorescent bacterial colonies on Kings B media amended with nalidixic acid; these colonies were present in no more than one culture plate per sample. Based on diagnostic tests, none of these fluorescent bacteria could be identified as P. syringae (see Table 3). P. syringae gives negative results for oxidase and arginine dihydrolase tests. All fluorescent isolates from field samples growing on nalidixic acid were positive for these two tests. Therefore, these isolates do not resemble the genetically engineered P. syringae developed by AGS.

Non-fluorescent bacteria capable of growing on media amended with nalidixic acid were common in all plant samples (Table 2). Bacterial isolates from field samples showed variation in their ability to incite a hypersensitive reaction in tobacco leaves and their ability to nucleate ice formation.

The positivae control samples (numbers 33-40) yielded many fluorescent colonies for all replications. The diagnostic test profile for an isolate of a genetically engineered deletion mutant of P. syringae supplied by AGS matched the expected test profile for P. syringae, and was identical to the test profile for isolates from control samples 33, 34, 37, 38, 39 and 40. Except for the hypersensitivity test on tobacco, the diagnostic test profile for an isolate of a genetically engineered deletion mutant of P. fluorescens supplied by AGS matched

Table 2. Presence of bacteria from plant samples on amended King's B media.

| Sampling Location | Sample # | Sample Type | King's B Media + Cycloheximide Amended with Antibiotics | |
|---|---|---|---|---|
| | | | Rifampicin | Nalidixic Acid |
| 1 | 1 | Mixed leaves | - | + NF |
| 1 | 2 | Mixed leaves | - | + F1 1 colony |
| 2 | 3 | Apricot leaves | - | + NF |
| 2 | 4 | Mixed leaves | - | + F1 1 colony |
| 3 | 5 | Mixed leaves | - | + NF |
| 3 | 6 | Mixed leaves | - | + NF |
| 3 | 7 | Mixed leaves | - | + NF |
| 3 | 8 | Mixed leaves | - | + NF |
| 4 | 9 | Blackberry leaves | - | + NF |
| 4 | 10 | Blackberry leaves | - | + NF |
| 5 | 11 | Bay-type leaves | - | + NF |
| 4 | 12 | Mixed leaves | - | + NF |
| 4 | 13 | Strawberry leaves | + NF | + NF |
| 4 | 14 | Strawberry blossoms | - | + F1 3 colonies |
| 4 | 15 | Strawberry leaves | + NF | + NF |
| 4 | 16 | Strawberry leaves | - | + NF |
| 4 | 17 | Strawberry leaves | - | + F1 2 colonies |
| 4 | 18 | Strawberry leaves | - | + NF |
| 4 | 19 | Strawberry blossoms | - | + F1 1 colony |
| 4 | 20 | Strawberry leaves | - | + NF |
| 4 | 21 | Strawberry leaves | + NF | + NF |
| 4 | 22 | Strawberry leaves | + NF | + F1 1 colony |
| 4 | 23 | Strawberry blossoms | - | + F1 1 colony |
| 4 | 24 | Strawberry leaves | - | + NF |
| 4 | 25 | Strawberry leaves | - | + NF |
| 4 | 26 | Strawberry leaves | - | + F1 10 colonies |
| 4 | 27 | Strawberry leaves | + NF | + F1 2 colonies |
| 4 | 28 | Strawberry blossoms | - | + NF |
| 4 | 29 | Strawberry leaves | - | + F1 2 colonies |
| 4 | 30 | Strawberry leaves | - | + F1 1 colony |
| 4 | 31 | Strawberry leaves | - | + NF |
| 4 | 32 | Strawberry leaves | + NF | + F1 1 colony |
| 6 | 33 | Strawberry leaves | + F10 | + F10 |
| 6 | 34 | Strawberry leaves | + F2 | + F10 |
| 6 | 35 | Strawberry leaves | + F10 | + NF |
| 6 | 36 | Strawberry leaves | + F10 | + NF |
| 6 | 37 | Strawberry leaves | + F10 | + F10 |
| 6 | 38 | Strawberry leaves | + F10 | + F10 |
| 6 | 39 | Strawberry blossoms | + F10 | + F10 |
| 6 | 40 | Strawberry blossoms | + F10 | + F10 |

\+ Indicates bacterial growth on the media after 48 to 72 hours.
\- Indicates no bacterial growth on the media after 48 to 72 hours.
F Indicates the presence of fluorescent bacterial colonies on the plates. The number after F indicates the number of plates with fluorescent bacteria. Plates with fluorescent bacteria had many colonies unless actual number of colonies is specified.
NF Indicates growth of only non-fluorescent bacteria on plates.

Table 3. Summary of characterization of fluorescent bacteria taken from King's B media amended with nalidixic acid or rifampicin.

| Sample Number | Selective Media | Fluorescence | Oxidase | Arginine Dihydrolase | Hypersensitivity on Tobacco | Ice Nucleation |
|---|---|---|---|---|---|---|
| Ps[a] | N[b] | + | - | - | + | - |
| 2 | N | + | + | + | - | - |
| 4 | N | + | + | + | - | - |
| 14 | N | + | + | + | - | - |
| 17 | N | + | + | + | + | - |
| 19 | N | + | + | + | - | - |
| 22 | N | + | + | + | - | - |
| 23 | N | + | + | + | - | - |
| 26 | N | + | + | + | - | - |
| 27 | N | + | + | + | - | + |
| 29 | N | + | + | + | - | - |
| 30 | N | + | + | + | - | - |
| 32 | N | + | + | + | - | + |
| 33 | N | + | - | - | + | - |
| 34 | N | + | - | - | + | - |
| 37 | N | + | - | - | + | - |
| 38 | N | + | - | - | + | - |
| 39 | N | + | - | - | + | - |
| 40 | N | + | - | - | + | - |
| Pf[c] | R[d] | + | + | + | - | - |
| 33 | R | + | + | + | + | - |
| 34 | R | + | + | + | + | - |
| 35 | R | + | + | + | + | - |
| 36 | R | + | + | + | + | - |
| 37 | R | + | + | + | + | - |
| 38 | R | + | + | + | + | - |
| 39 | R | + | + | + | + | - |
| 40 | R | + | + | + | + | - |

a. Ps = Isolate of genetically engineered deletion mutant of P. syringae supplied by AGS.
b. N = Kings B medium amended with nalidixic acid.
c. Pf = Isolate of genetically engineered deletion mutant of P. fluorescens supplied by AGS.
d. R = Kings B medium amended with rifampicin.

the test profile for isolates from positive control samples 33 through 40, and fit the expected test profile for P. fluorescens. For six of the eight positive control samples, numbers 35 through 40, genetically engineered P. syringae or P. fluorescens applied to strawberry leaves alone or in combination were isolated and correctly identified (see Table 4). For samples 33 and 34, although only P. syringae was intentionally applied, bacteria matching the expected profiles of both P. syringae and P. fluorescens were isolated from strawberry foliage.

## DISCUSSION

It can be concluded from diagnostic test results that bacterial isolates from field samples did not possess the distinguishing characteristics expected for genetically altered P. fluorescens or P. syringae. No bacteria were detected from field samples from Monterey County which matched the expected characteristics of genetically engineered Pseudomonas species produced by AGS. In contrast, except for the hypersensitivity test, Pseudomonas species with characteristics that fit the expected profile for genetically engineered bacteria were consistently isolated from positive control samples.

It is highly unlikely that genetically engineered bacteria with characteristics of the AGS product had been applied to the Monterey County field locations during the time period immediately preceding vegetation sampling. It is yet to be determined how long genetically engineered Pseudomonas species can survive and at what level the population of surviving cells will be maintained. This information along with a consideration of limits of detection for the enumeration method employed (dilution plating on selective media) would allow a more definitive statement to be made concerning the timeframe for detectability of genetically engineered bacteria following field application.

Table 4. Genetically engineered, antibiotic resistant bacteria applied and isolated from control samples.

| Sample Number | Antibiotic Resistant Organism Applied [a] | Antibiotic Resistant Organism Isolated |
|---|---|---|
| 33 | Ps | Ps + Pf |
| 34 | Ps | Ps + Pf |
| 35 | Pf | Pf |
| 36 | Pf | Pf |
| 37 | Ps + Pf | Ps + Pf |
| 38 | Ps + Pf | Ps + Pf |
| 39 | Ps + Pf | Ps + Pf |
| 40 | Ps + Pf | Ps + Pf |

a. Ps = Pseudomonas syringae
Pf = Pseudomonas fluorenscens

Deviation from expected results for the hypersensitivity test occurred only for isolates from positive control samples which otherwise fit the expected profile of P. fluorescens. Variability in hypersensitivity test results has been reported for Pseudomonas species in general (1) and P. fluorescens in particular (5). This variability is determined by plant temperature before and after inoculation, light conditions following inoculation and the concentration of viable cells used for inoculum.

Given the fact that the hypersensitivity test for laboratory control cultures were conducted approximately three weeks after hypersensitivity tests for positive control isolates from greenhouse inoculated strawberry foliage, the difference in test results was most likely due to different environmental conditions at the time of testing. The variability which is possible in hypersensitivity tests even for saprophytic bacteria requires that hypersensitivity tests for laboratory control and field isolates be conducted simultaneously under uniform conditions.

Rifampicin resistance appears to be a more selective marker for use in detecting bacteria from a mixed microbial population than nalidixic acid. Bacterial colonies were common on nalidixic acid amended media. Nalidixic acid for use as a antibiotic resistance marker may enhance selectivity when combined with another form of antibiotic resistance, but when used alone appears to be less selective than desired for rapid field monitoring since additional tests may be needed to characterize isolates able to grow on less selective media.

In general, the two genetically engineered Pseudomonas species were correctly identified whether applied alone or in combination to strawberry leaves or

flowers. Sample number 33 and 34 were the only exceptions; from these samples bacteria matching the expected profile for both Pseudomonas species were isolated when, according to information from AGS, only one species had been applied. It is difficult to determine with certainty the cause of this discrepancy, but it is likely that both species were present on the vegetation at the time of sampling. In any case, Pseudomonas species which were applied to positive control leaf and blossom samples were in all cases detected and correctly identified.

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APPENDIX I

STATE OF CALIFORNIA
DEPARTMENT OF FOOD
AND AGRICULTURE

Special Project
CHAIN OF CUSTODY RECORD
*(use ball point pen only)*

ENVIRON. MONITOR. & PEST MGMT.
ENVIRON. HAZARDS ASSESSMENT
1220 N STREET, ROOM A-149
SACRAMENTO, CA 95814

| Study # | Sample # | Date On | | | Time On | Date Off | | | Time Off | Person Collecting | Location | Sample type | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mo | Day | Yr | | Mo | Day | Yr | | | | | |
| | X | | | | | | | | | | | | |
| 1 2 3 4 5 | 6 7 8 9 | 10 11 | 12 13 | 14 15 | 16 17 18 19 | 20 21 | 22 23 | 24 25 | 26 27 28 29 | 30 31 | 32 33 | 34 35 36 | 37 38 39 40 |

| | Chemist | Lab # |
|---|---|---|
| | | |
| 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 | 75 76 | 77 78 79 80 |

Partner: Location:

Remarks:

**Lab Results:** Save extracts

Chemist: Date:

**KEY**

Col. 1
S = Spike

Col. 2
* = Split

Col. 3
X = to delineate special project.

Col. 31-32: (Location)
01 =
02 =
03 =

Col. 33-35: (Sample Type)
Hiv = Hivol
Lov = Lo-vol
Wat = water
Soi = soil
Lea = leaf
Fru = fruit

| | | |
|---|---|---|
| Relinquished for Lab by: (Signature) | Date/Time | |
| Received by: (Signature) | Relinquished by: (signature) | Date/Time |
| Received by: (Signature) | Relinquished by: (Signature) | Date/Time |
| Received by: (Signature) | Relinquished by: (Signature) | Date/Time |
| Received by: (Signature) | Relinquished by: (Signature) | Date/Time |
| Received for Lab by: (Signature) | Date/Time | Lab # |



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