METABOLISM, NUTRITIONAL EFFECTS, AND MUTAGENESIS OF CRYSTAL VIOLET DECOLORIZATION BY A BIOFUNGICIDE AGENT PSEUDOMONAS PUTIDA STRAIN M-17

by

Fred Talmadge McCuistion

Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the
degree of

MASTER OF SCIENCE

in

Crop and Soil Environmental Sciences

APPROVED:

C. Hagedorn
Chairman

D. Berry

E. Stromberg

February, 1991

Blacksburg, Virginia

ABSTRACT

A strain of Pseudomonas putida that exhibited seedling disease control on cotton was among 5000 strains examined for unique properties that could be used to selectively recover genetically engineered pseudomonads from environmental samples. One isolate (M-17) was found to produce a red halo around single colonies grown on media containing 10 mg/l crystal violet. This decolorization reaction was constituitively produced when the growth medium contained glucose and asparagine, but was inhibited by the substitution of ammonia, nitrate or urea for the amino acid nitrogen source. However a different medium containing succinic acid and using ammonia as the sole nitrogen source was found to induce the reaction. Factors that did not affect the decolorization reaction included temperature (14-30° C) and pH (3-8). Cultures of M-17 grown in broth containing crystal violet were able to decolorize still broth solutions, but not when incubated on a shaker (150 rpm). Stationary cultures formed a red precipitate. Efforts to characterize the precipitate revealed that it was soluble in polar organic solvents and insoluble in non-polar organic solvents. Thin layer chromatography revealed the presence

of six bands that possibly represented various demethylated forms of crystal violet. Chemically derived mutants (cry-) did not produce a red halo but a clear, colorless region surrounding bacterial growth was observed. A greenhouse study demonstrated that strain M-17 provided protection against fungal disease as shown by plant stands on cotton (67% stand) equivalent to the commercial fungicides (70% stand) and significantly improved over the unammended control (38% stand).

ACKNOWLEDGEMENTS

I wish to thank my advisor Dr. Hagedorn for his helpful comments, directions, and efficiency of operation. I would like to thank my committee members Dr. Berry and Dr. Stromberg for additional help they provided in reviewing the manuscript. I would like to thank Norma Nelson, Phil Keating, Claude Kenley and Lloyd Flinchum for their technical assistance. I express my deepest appreciation to my parents for the Godly instruction, guidance, patience and encouragement they have given me not only in pursuing this work, but throughtout my life. Lastly I thank God both as my Savior and for giving me the abilities that He has. I pray that I may use them all for His glory.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

Chapter I

INTRODUCTION

During the germination of seeds, many different bacteria and fungi become associated with the seedling roots. Some of these microbes have little effect on the plant, while others are virulent pathogens and cause pre- or post-emergence damping-off disease on a large number of crop species.

Traditionally these pathogens have been dealt with in several ways. The losses were either accepted, Fusarium wilt resistant varieties were selected, or crop rotation was practiced to limit losses by prohibiting build up of pathogenic populations. In this century, as agriculture became more mechanized and monocultural, the application of chemicals to control seed and seedling pathogens became the dominant practice. However, it was noted that in some fields where monoculture was practiced, the severity of disease became less acute after several years (18,83). It was also discovered that bacterial populations of fluorescent pseudomonads were elevated in these

"suppressive" soils (17-20,28,84).

The observation that the natural balance of bacteria and fungi in the root zone could be altered led to the discovery that plant growth and yields could be improved in both root and seed crops by adding particular strains of Pseudomonas fluorescens Migula and Pseudomonas putida (Trevisan) Migula (13,15,16,45,48,72,85). The discovery leading to biological control of plant pathogens is an important nonchemical alternative to pest control. This concept, when coupled with the growing awareness of the environmental effects of applying chemicals, increasing resistance of pathogens to available chemicals, and the high costs of research to develop new chemicals, has reawakened interest in both reduced usage of chemicals and organic farming. Biological control agents have opened opportunities for the development of products based on the natural control of plant pathogens(47).

Crop loss due to pathogens is a large problem evidenced by the monetary resources spent on applying fungicides to minimize damages caused by fungal diseases, the research into developing plant resistance and biological control where chemicals are not effective. For example, yield losses in cotton, due to reduced stand and vigor caused by seedling disease, have been estimated from one to six

percent over a thirty year period which is equivalent to a loss of $40 million dollars annually. The sales of fungicides to control these pathogens on U.S. cotton alone are $50 million per year.

The first biological disease control products were based on the isolation and identification of naturally occurring bacterial strains that are the most effective in the inhibition of soil-borne fungal pathogens Rhizoctonia solani,(Kuehn) and Pythium ultimum (Trow) (47). Usually, the biocontrol strains are placed in a granular peat carrier and applied in-furrow to cotton seeds as an alternative to fungicides (38,39). Results with one biocontrol product, show that this method has been successful in 85% of the applications, while low soil pH contributed to a majority of the failures in the field (51).

A new generation of biocontrol agents may be possible through improved selections of microbial strains that provide protection at lower soil pH values. Other products may be the registration of these naturally occurring strains for additional crops or against other pathogens that these strains have been found to inhibit (51,63). Because fluorescent pseudomonads are efficient root-surface colonizers and some have proven beneficial to plant growth, these microbes have been targeted for genetic engineering

experiments that would incorporate genes for the production of toxins specific to a particular pathogen(66,67). These second generation products would extend the range of organisms inhibited by pseudomonads beyond their natural cababilities.

Concerns have been expressed by the public and governmental agencies that genetically engineered bacteria may have an adverse effect on the balance of soil microbes and may have negative long term effects. Therefore it is of interest to be able to track and selectively recover released organisms (53), or to construct strains that would die off once the level of substrate was spent(61). Traditional methods of tracking bacteria involve antibiotic resistance as genetic markers. However, these methods are of limited use because of the following problems (1) there exists a small but unacceptable level of spontaneous mutations, (2) unknown reversion frequencies and (3) gene transfer in resistance markers. In response to this uncertainty, the Monsanto Corporation (St. Louis) has developed a system by which the genes coding for lactose utilization can be transferred to pseudomonads. This genus of bacteria does not posses the ability to utilize lactose (10). These genes also code for an enzyme system capable of cleaving the dye 5-bromo-4-chloro-3 indoyl-ß-D-

galactopyranoside (X-Gal) resulting in the production of blue colored colonies. These blue colonies would constitute the genetically engineered strain. The ability of pseudomonads to utilize lactose and cleave X-gal might also confer a competitive advantage in certain environments such as a dairy or dairy products (22).

An alternative trait which may be simpler, safer and as efficient in selectively recovering genetically engineered microbes is the decolorization of the dye, crystal violet, from purple to red. This trait appears to be unique to one naturally occurring strain (P. putida M-17) and has not been explored further. The research reported in this thesis provides additional information on this strain and is an initial step in identifying the genetic elements necessary to transfer this trait to a recipient strain and thereby confer the ability to decolorize crystal violet.

Chapter II

LITERATURE REVIEW

A. Factors Affecting Fluorescent Pseudomonads as Biological Control Agents of Plant Pathogens

Strains of the bacterial species Pseudomonas fluorescens and Pseudomonas putida have been characterized that promote plant growth and inhibit soil-borne plant pathogens. Mechanisms by which these bacteria benefit the plant include: colonization of the root surface; stimulation of plant defense mechanisms; and production of antimicrobial compounds (antibiotics and siderophores).

1.Colonization of root surface

Pseudomonads must colonize the root surface in order to interact with the plant and other soil microflora. Although strain dependent, pseudomonads are highly capable of colonizing the root surface (57). The mechanisms for this process, while not fully understood involve rapid bacterial growth (49), motility (24,40), chemotaxis to root exudates (37,74), water matric potential and movement through the soil profile (14,28,68), osmotolerance (57), bacterial surface receptors which recognize a root surface

polysaccharide or a glycoprotein (agglutinin) (3-8,23,29,43,55,70,81), the presence of bacterial pilli or fimbrae (79,80,89,90) and the presence of divalent cations (28-30,41,55).

2. Stimulation of defense mechanisms

The colonization of the root surface by pseudomonads has been shown to stimulate a higher level of lignin production in roots of beans Phaseolus vulgaris under hydroponic conditions. It is believed that lignified cells delayed the early stages of infection by the fungal pathogen Fusarium solani (Mart.) Appel & Wr. (7,52). Additionally, higher levels of peroxidase were produced, which leads to enhanced production of extensin and lignin. As a result of peroxidase catalyzed reactions, quinones, (71) and phenoxyl radicals with antimicrobial activity were also produced induced (1,2).

3. Competition for nutrients

It has been demonstrated that selected strains of pseudomonads lower the incidence of the fungal pathogen

Fusarium spp. by competing with chlamydospores for nutrients(31). This was based on observations that Fusarium chlamydospores did not germinate when strains of select pseudomonads were present, and previously established evidence that chlamydospore germination was affected by exogenous nutrients (73). The role of nutrient competition was further confirmed by demonstrating that no bacterial produced lytic enzymes were involved in the reduction of disease. The role of bacterial iron-chelating compounds in reducing disease incidence was precluded by the use of media that allowed for sufficient iron availability (73,75,83).

4. Antimicrobial compounds

a. Siderophores

Experiments have shown that pseudomonads which inhibit Pythium spp., Fusarium spp. and Gaeumannomyces spp. and some deleterious bacteria do so by the production of iron III transport agents designated as siderosphores (13). Siderophores are produced under iron limiting conditions and tightly complex with iron III making it less available to

other microorganisms and thereby inhibiting their growth (59). Furthermore, the beneficial pseudomonads produce siderophores that cannot be utilized by other pseudomonads, thus implying a specificity in siderophore recognition by an outer membrane receptor (59). Due to their large molecular weight (1000 M.W.), siderophores must be actively transported by means of a receptor protein in the outer membrane (59). The role of siderophores in inhibiting pathogens via iron starvation was confirmed by adding iron in sufficient quantity to saturate the siderophores produced and thereby negate their effect on fungal disease incidence (72,73). Additionally, mutants unable to produce siderophores could not inhibit the pathogen (59).

Additional research on siderophores has been conducted to elucidate the physical structures, the genetics of siderophore production and the biochemistry of the outer membrane siderophore receptor protein.

The structure of siderophores appears to conserve three bidentate iron-chelating lignands. The molecule contains a short peptide chain that is linked to a fluorescent quinoline derivative (77,78) (Fig. 1). Variations in the peptide chain length and amino acid constituents are common and appear to play a role in the ability of one pseudomonad to recognize and assimilate the siderophore of another.

Additionally the presence of D-amino acids may confer a resistance to the metabolic breakdown of the structure which may account for the relative persistence of these compounds in the rhizosphere(54).

There are several reports of genetic analysis of siderophore production(58-60). The results for different strains vary from three gene clusters containing five genes to four gene clusters containing twelve genes (59), to five gene clusters containing seven genes (60). Below is a summary of one of the reports. A fluorescent pseudomonas strain (B 10) was found to have a siderophore termed pseudobactin that consisted of a linear hexapeptide: L-Lys-D-threo-B-OH-Asp-L-Ala-D-allo-Thr-L-Ala-D-N-OH-Orn linked via an amide bond to a fluorescent quinoline derivative. The strain was mutagenized by nitrosoguanidine, ethylmethane sulfonate, and ultraviolet light. Fluorescent negative mutants were individually complemented by mating with a gene bank constructed from the broad host range conjugative plasmid pLAFR1. The results showed that eight recombinant plasmids complemented 154 of 157 mutants. Based on complementation patterns, four gene clusters containing up to twelve genes were considered necessary for pseudobactin synthesis. This was deemed a reasonable number based on the

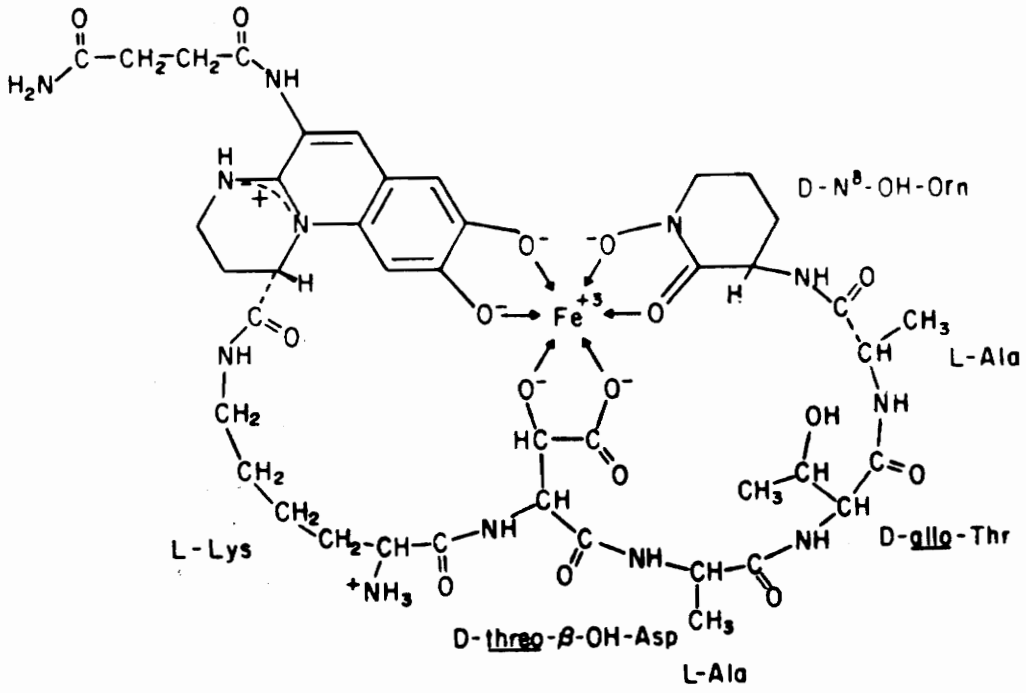


Fig. 1. Chemical structure of a siderophore isolated from Pseudomonas putida.

necessity of isomerases required to synthesize unusual amino acids. The synthesis of the quinoline derivative was not reported (59).

To extend the understanding of how pseudomonads inhibit soil-borne fungal pathogens and compete with other Pseudomonas strains, the ability of a particular isolate to produce and utilize a siderophore that made iron unavailable to competing strains was studied. This ability revolves around the receptor which recognizes the siderophore-iron complex. To examine this concept, the gene for the siderophore receptor from a Pseudomonas (B 10) which inhibited the growth of two other pseudomonads (A124,A225) was identified by transposon mutagenesis. A plasmid, pJLM 300, was constructed which contained a functional 2.4 kilobase region coding for the 85 kDa B 10 siderophore receptor. This plasmid was inserted into the two strains whose growth was normally inhibited by Pseudomonas B 10 or its siderophore pseudobactin. The recipient strains, now containing the gene for the outer membrane receptor for B 10, were no longer susceptible to iron starvation by pseudobactin because they were able to transport it (59).

b. Antibiotics

Pseudomonads have been shown to produce the antibiotics pyrrolnitrin, pyoluteorin, and a dimer of phenazine carboxylic acid. The production of antibiotics has also been postulated as a mechanism for biological disease control of fungal pathogens (35,36,38,39,42,69). Although many pseudomonads produce both antibiotics and siderophores, the experiments were carried out *in vitro* and with purified forms of the antibiotic. Pathogens inhibited by these antibiotics include *Rhizoctonia solani*, *Pythium spp.*, *Erwinia carotovora* (L.R. Jones), *Verticillium dahliae* Kleb., *Alternaria sp.*, and *Thielaviopsis basicola* (Berk.& Br.) Ferr. Other reports of biological control are for *Phytophthora sp.* and *Alternaria sp.* and *Fusarium sp.* (Table 1). For example, *in vitro* experiments demonstrated that *P. fluorescens* strain HV37a inhibited the growth of *Pythium ultimum* (36,42). In addition, mutants of HV37a which did not produce the antifungal compounds were reduced in their capacity to protect cotton seedlings from *Pythium ultimum*.

To develop a better understanding of the synthesis of pseudomonad antibiotics, the genetics of antibiotic production were studied by the use of chemical mutagenesis followed by recombinational restoration of the HV37a mutants

Table 1. Plant Pathogens, the diseases they cause, their host range, and the degree of biological control by a particular Pseudomonad.

Phytopathogen	Disease	host range	Pseudomonas sp. strain	Effect of Biocontrol
Fusarium solani	Vascular wilt	beans, peas, cotton, cucumber, melons	P. putida A12	40% disease control in cucumber. Straight 8.
Gaeumannomyces graminis var. tritici	take all	wheat, barley	P. fluorescens	27% yield increased non-fumigated soil.
Pythium sp.	damping off, crown rot, feeder root disease	many crops	P. fluorescens HV37a	13% sugar increase in sugar beets
Rhizoctonia solani	damping off, root rot	many crops	P. fluorescens	comparable to or better than fungidical control
Thielaviopis basicola	black root rot	tobacco, cotton beans & others	P. flourescens PF-5	"highly inhibits at 25 µg/ml in vitro."
Verticillium dahliae	wilt	cotton & many other plants	P. flourescens PF-5	"highly inhibits at 25 µg/ml in vitro."
Erwinia carotovara	soft rot	potatoes, carrots, tomatoes	Pseudomonas sp. B 10	70 % increase in tuber yield.

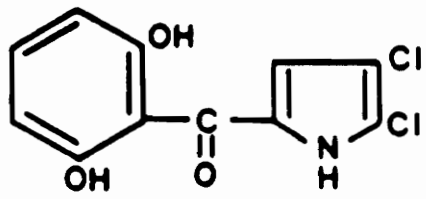
with wild type DNA. Based on co-synthesis tests, three classes of mutants were found. A library of HV37a chromosomal DNA was then constructed in a cosmid and individual cosmids were tested for antibiotic producing genes by insertion into mutant strains and examining the strains for complementation. Each cosmid was then subjected to transposon mutagenesis to localize the antibiotic genes and to determine whether the cosmid contained more that one gene necessary for antibiotic synthesis. The results of this experiment show that at least three separate genomic regions containing a total of five genes are required for antibiotic production (36,42). A separate experiment using Pseudomonas fluorescens strain PF-5 identified an antibiotic pyrrolnitrin (3-chloro-4-[2'-nitro-3'-chlorophenyl]-pyrrole) based on R_f values, NMR and mass spectral data. This antibiotic was highly inhibitory to the fungus Rhizoctonia solani based on survival of cotton seedlings in flats containing the pathogen(38). The efficacy of both the bacteria and the purified antibiotic were examined in these tests. The treatment of seed with 6.6 g pyrrolnitrin/seed resulted in plant survival rates of 70% compared to 13% for untreated seed. Survival of cotton seedlings was 79% when treated with 2 ml of bacterial culture (7×10^8 cells/ml)

compared to 30% for untreated seed. Additionally *in vitro* tests showed that the antibiotic (25 g/ml) was highly inhibitory to *T. basicola*, *Alternaria sp.* and *V. dahliae*, and inhibited the growth of *Fusarium sp.* by 30%. *P. ultimum* was not inhibited (38).

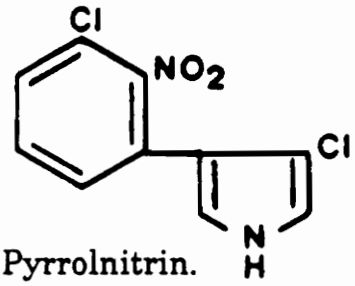
A different antibiotic was isolated and characterized from *P. fluorescens* strain 2-79 which, under *in vitro* conditions, inhibited *Gaeumannomyces graminis* (Sacc.) von Arx & Oliver var. *tritici* Walker, *Rhizoctonia solani* and *Pythium sp.*. The antibiotic, based on UV, infrared NMR, mass spectral and elemental analysis, was proposed to be a dimer of phenazine carboxylic acid (Fig. 2) (35).

5. Genetic engineering

The possibility of using plant beneficial strains of *Pseudomonas sp.* as targets for genetic engineering has been explored in detail over the past decade. The δ-endotoxin protein of *Bacillus thuringiensis* Berliner subsp. *kurstaki* (which was effective against lepidopteran insect larvae) has been expressed by a genetically engineered strain of *P. fluorescens*, (Ps 112-12a)(66,67). The tobacco budworm (*Heliothis virescens* Boddie) was chosen as the subject of control by the genetically engineered strain. The results



Pyoleutorin.



Pyrrolnitrin.

Fig. 2. Structure of several antibiotics produced by Pseudomonas sp. inhibitory to the fungal plant pathogens Gaeumannonyces graminis var. tritici, Pythium sp. and Rhizoctonia solani.

of the larvae feeding on the toxin containing bacteria were a significant reduction in pest levels, but increased insect resistance to the toxin over several generations was noted. It was postulated that resistance to the toxin was developed due to a continuous exposure to the toxin, unlike field conditions. Additionally, variations within the insect population, migration, dispersal, alternate hosts, generations per year, frequency of application and total acreage were all listed as possible factors affecting the development of resistance similar to chemical pesticides(76). While not eliminating this method of pest management, this experiment, if representative of other results, will make development of genetically engineered bio-control agents a more complex undertaking.

When the literature available on this topic is considered, it is clear that through efficient colonization of the root surface, various Pseudomonads are capable of adequately protecting seedlings of important crop species against major fungal pathogens, Alternaria sp., Fusarium sp., Gaeumannomyces graminis, Pythium spp., Rhizoctonia solani, Thielaviopsis basicola, and Verticillium dahliae, Phytophthera spp. and the bacteria Erwinia carotovara. This results in increased crop yields or yields equivalent to those achieved by chemical controls. The two most

significant mechanisms for this attribute are thought to be the production of iron-chelating agents called siderophores which inhibit fungal growth under iron limiting conditions and the production of antibiotics that inhibit the growth of the fungi. Additionally, pseudomonads that efficiently colonize the root surface are excellent targets for genetic manipulation to provide protection against a variety of plant pathogens and pests. Other approaches that may promote plant growth through enhanced mineral availability or production of plant growth regulators is an area of potential benefit that has not yet been well characterized or explored.

B. Tracking Genetically Engineered Pseudomonads

In an effort to develop a simple phenotypic marker to trace a genetically engineered strain of Pseudomonas sp. released into the environment, the genes for lactose utilization were transferred from Escherichia coli (Migula) Castellani and Chalmers to a fluorescent pseudomonad (27). Pseudomonads in general cannot utilize lactose as a carbon source (10). The genes conferring the ability to utilize lactose also confer the ability to cleave the dye X-Gal which turns from clear to blue when cleaved. Therefore a

genetically engineered pseudomonad containing the lactose utilization genes could be followed through the environment by placing a dilution of a field sample onto Pseudomonas-specific media containing lactose and X-Gal. The engineered organism would be the only bacterial colony that would produce a blue halo around the colony, thus providing a relatively simple method for identification and tracking a genetically engineered microorganism (GEMS) in a released environment.

An additional phenotypic marker that could provide a selective recovery mechanism for GEMS is the production of a red halo around a bacterial colony on a medium containing the dye crystal violet (Fig 3).

C. Crystal Violet Metabolism

Crystal violet (CV) is a toxic triphenylmethane dye (Fig. 4) used in the textile industry and as an antibacterial agent in poultry feed. It is also used as a diagnostic aid in human and veterinary medicine. Wastewater treatment facilities are unable to remove triphenylmethane dyes from contaminated wastewater, thus often contributing a persistent, mutagenic, carcinogen to aqueous habitats and human food chains (12). It is therefore of great interest

to study the metabolism of this dye in both mammals and microorganisms. There are reports of CV metabolism in bacteria, fungi, and mammals (12,50,62,86-88). The metabolism described in all these reports is simple N-demethylation although, due to the diversity of such sources, it is not unrealistic to think that several different mechanisms and regulatory systems are involved in CV metabolism. However, no information has been reported concerning the nutritional regulation of CV metabolism.

1. Bacteria

The biodegradation of CV by cultures of Pseudomonas pseudomallei (Whitmore) 13NA was reported (86-88). The disappearance of CV based on decreased U.V. absorption over time was interpreted as metabolism of the dye. Additionally dichloromethane was used to extract the solution containing the culture and thin layer chromatography of this extract produced a spot, not for crystal violet (R_f 0.1), but for an unidentified metabolite at R_f 0.6 (87). A more recent study reported that when CV concentration was 5×10^{-5}M, the half decolorization time by broth cultures of the bacteria was 61 hours as measured by the decrease in the visible spectrum. The authors did not further discuss the metabolites (86).

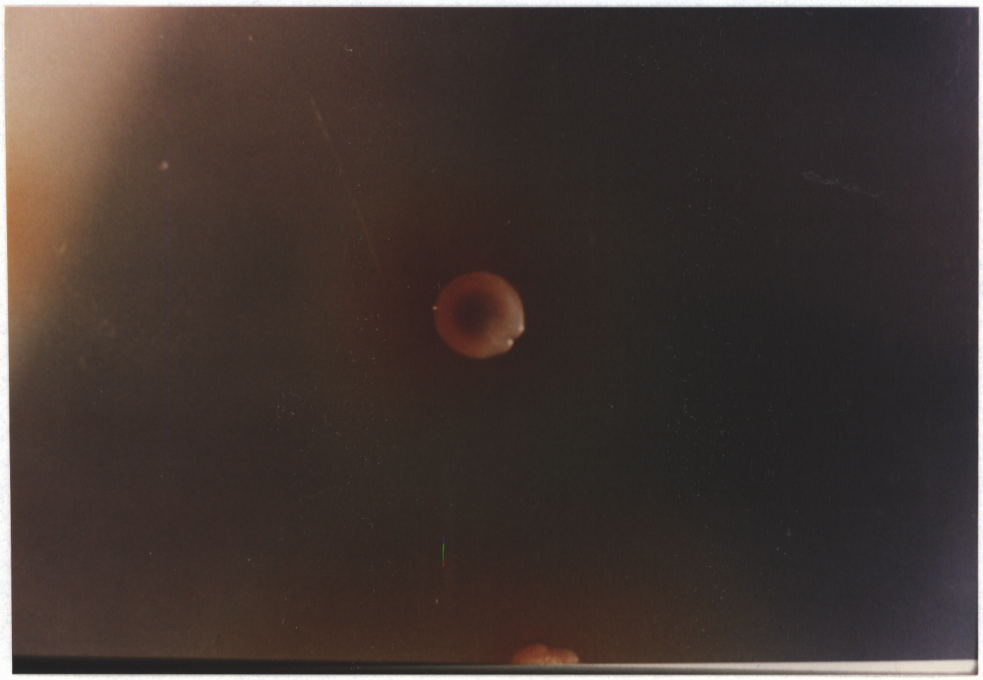


Fig. 3. Halo formation resulting from decolorization of crystal violet by Pseudomonas putida (M-17) on PF medium.

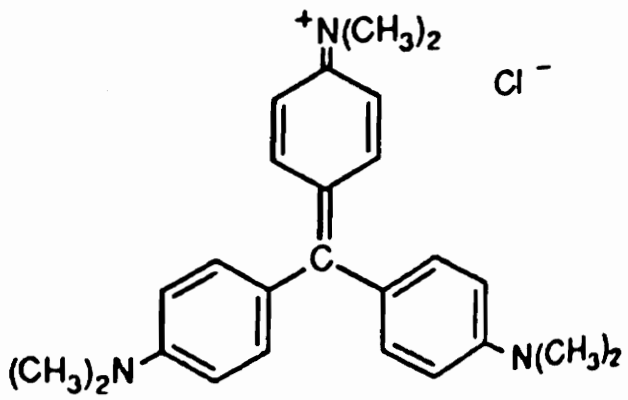


Fig. 4. Chemical structure of crystal violet (aka. gentian violet, hexamethylpararosaniline).

2. Fungi

The biodegradation of CV by the white rot fungus Phanerochaete chrysosporium Karst. was reported (12). Degradation was demonstrated by using HPLC to measure CV metabolite formation in cell free methylene chloride extracts or by using UV-vis absorption to assay the concentration of CV in extracellular culture samples. Thin layer chromatography was used to separate three metabolites (penta-, tetra-, and tri-methylpararosaniline) that were identified by Rf values similar to analytical standards of these compounds. These standards are not commercially available but are obtained by thin layer chromotography of methyl violet which contains them. Additionally, a purified ligninase catalyzed N-demethylation of CV, thus establishing that biodegradation was dependent upon the lignin degrading system. However, an additional, and as of yet unidentified, mechanism for degrading CV was suggested because non-ligninolytic cultures also were able to degrade CV.

Crystal violet biodegradation by cultures of the oxidative red yeast Rhodotoraula sp. was identified by

decreased visible absorption in broth cultures and on agar plates as clear zones around colonies of the yeast. The broth cultures completely degraded 10 mg/l of the dye in only four days. To confirm that the disappearance of the dye was not due to absorption by the yeast biomass, the cells were sonicated and extracted with 70% ethanol. The extract showed no absorbance by CV (50).

Although the metabolites were not identified, colonies of the fermentative yeast Saccharomyces cerevisiae Meyen became deep purple in color ten days after incubation, without forming a clear zone, which suggested bioconcentration of the dye (50).

3. Mammals

Metabolism of CV by uninduced liver microsomes obtained from mice, rats, hamster, guinea pig, and chickens was examined in vitro. Metabolites isolated by solvent extraction were identified by HPLC by comparison with authentic standards prepared by unambiguous synthetic routes. The major metabolites of CV (hexamethylpararosaniline) were identified as penta- and tetra-methylpararosaniline (62). The demethylation of dimethylamino substituents was reported to involve

hemoprotein of the mixed function oxidase system (11).

D. Mutagenesis

As no mutations or genetic analysis of the metabolism of crystal violet by P. putida have been reported, examples of mutagenesis and genetic analysis of traits are briefly reviewed. There are several approaches which may be used to elucidate the number, sequence, and regulation of genes involved in controlling and affecting a particular trait. The methods most commonly employed are chemical or transposon mutagenesis (33,65).

1. Chemical

Chemical mutagenesis usually involves either ethylmethanesulfonate (EMS) or nitrosoguanidine (Ntg) as agents which create random mutations in organisms. Several workers have used chemical mutagenesis to explore the production of siderophores by pseudomonads (25,44,46,56). Mutants of Pseudomonas sp. B 10 deficient in the ability to produce siderophores were obtained by incubating broth cultures (2 x 10^8 cells/ml) of the bacteria with media containing either EMS (4% for 1.0 h at 30° C) or Ntg (2

mg/ml for 4.0 h at 30° C). Mutants were detected by a loss of fluorescence. It was presumed that these mutants lost the ability to fluoresce because one or more of the genes involved in the production or expression of the bacterial siderophore was rendered non-functional by the mutagen. The next step was to construct a "library" or gene bank of the wild type strain. This is accomplished by subjecting DNA isolated from the wild type strain to a particular restriction enzyme which cuts the DNA into fragments about 10-35 kb in length. These fragments were integrated into the cosmid pLAFR1. This cosmid is a plasmid or small extrachromosomal DNA molecule capable of replication within the host organism. If a large enough number of cosmids are made, the entire wild type genome will be represented on these cosmids. Then, via a tri-parental mating of organisms, (the E. coli containing the cosmid, a helper [E. coli containing pRR2013], and the recipient [a mutant lacking in the production of its siderophore]) the cosmid containing DNA from the wild type strain (B 10) is inserted into the mutant strain. If the DNA inserted complements or contains the gene in which the mutation occurred, the production of the siderophore is restored. By determining the number of different cosmids which complemented the mutants, the number of genes controlling

the trait may be found. Additionally based on DNA sequence overlap, the proximity of the genes to one another may be determined (21,65).

2. Transposon

A second method of identifying the gene(s) responsible for a trait is to use transposon mutagenesis. The most commonly used transposable element (Tn5) is a 5700 base pair capable of random, usually single site, insertion into DNA. Two inverted repeats at either end of Tn5 contain the requiste genes and regulatory elements for transposition. In addition, successful insertions are identified by resistance to kanamycin which is conferred upon the recipient.

The transposable element works by inactivating the gene where it is inserted. Additionally, based upon the known DNA sequence of Tn5 it is easily identified within the recipient's genome. Therefore in desirable mutant phenotypes, the regions flanking the Tn 5 insertion will contain at least one of the genes required for that phenotype.

The procedure used to introduce Tn5 usually involves a

triparental mating. The three bacteria involved are 1) an E. coli strain carrying the transposable element on a plasmid; 2) an E. coli containing the mobilization plasmid pRK2013 and 3) the recipient strain. The pRK2013 plasmid mobilizes into the E. coli strain carrying the transposable element and provides proteins required for the mobilization of the plasmid containing the Tn5 element into the desired recipient strain. Once inside the recipient strain, the transposable element integrates into the resident genome through homologous recombination. After the mating procedure, all the strains are transferred to media which, through its composition, selects for growth of recipient organisms containing the Tn5 element (usually based on kanamycin resistance). Successful transformants are then transferred to media designed to select for desirable mutants (26).

Chapter III

MATERIALS AND METHODS

A. Factors Affecting Crystal Violet Metabolism

A study was initiated to determine how P. putida strain M-17 effects the color change and how this property is regulated. A series of experiments was designed to determine if: (1) CV induces the decolorization; (2) CV can serve as the sole carbon or nitrogen source; (3) alternate nitrogen or carbon sources affect CV degradation; (4) CV degradation is pH or temperature dependent; and (5) any additional factors affect CV degradation and whether alternate dyes could substitute for CV.

1. Inducibility by crystal violet

The original medium for fluorescent pseudomonads on which CV discolorization was observed is designated PF (34) (Table 2). To determine if discolorization is induced by either the presence of crystal violet or by components in the medium, a single heavy streak of P. putida M-17 was made on PF medium without crystal violet.

(BLANK PAGE)

TEXT CONTINUES ON FOLLOWING PAGE

Table 2. Medium for fluorescent pseudomonads (PF).

component	g/l
KH_2PO_4	0.5
$NaHCO_3$	1.0
Asparagine	1.0
Glucose	2.0
$MgSO_4$ $7H_2O$	0.1
$FeCl_3$	0.001
pH 5.5	
crystal violet	0.03 - 0.005

After 48 h incubation at 28°C in darkness, medium at several locations adjacent to, but not including any bacterial cells, was aseptically removed and transferred to PF medium containing CV (20mg/ml) and the occurence of any reaction was noted 24 h after transfer.

2. Utilization of crystal violet as an energy source

In an effort to determine if crystal violet could be used as the sole carbon and energy source or the sole nitrogen source, experiments were conducted where either the carbon or nitrogen sources were removed from the PF medium and CV was added at a concentration of 30 mg/l. In both experiments, M-17 was streaked onto the medium and a dilution series (10^{-5}-10^{-8}) was also plated. The cultures were incubated in darkness at 28°C and observed after 1, 2, 7 and 28 days for growth.

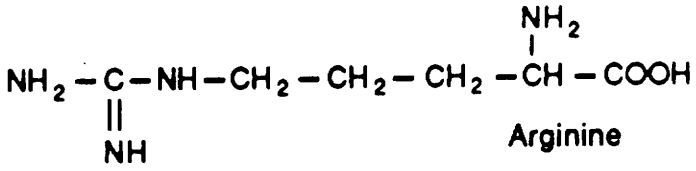
3. Nutritional effects

To examine regulation of CV decolorization, various nitrogen sources were evaluated. The normal PF medium contains asparagine (1.0 g/l) as the sole nitrogen source.

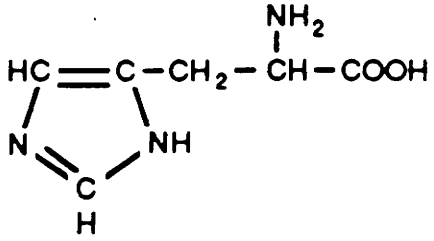
To determine if a minimum level of asparagine was required to induce the reaction, the level of asparagine was altered (0.2, 0.4, 2.0, 4.0 g/l) and the plates were scored for halo size and color 48 h after streaking and incubation in the dark at 25°C. To determine the effect of the amino acid nitrogen source, a total of twelve amino acids were substituted for asparagine (each at 1.0 g/l). The amino acids were: arginine; aspartic acid; cysteine; glycine; glutamine; glutamic acid; histidine; lysine; proline; threonine; tryptophan; and tyrosine (Fig. 5). M-17 was streaked onto the various media and the cultures were incubated in the dark at 25°C and observed every 24 h for 7 days. A third experiment substituted NH_4^+ , NO_3^- , or urea for asparagine as the sole nitrogen source, and the plates were scored 48 h after incubation in darkness at 25°C.

To determine if inorganic nitrogen could inhibit the decolorization reaction, asparagine was used at a reduced concentration (0.2 g/l) and either ammonia, nitrate, or urea in concentrations ranging from 0.1 - 2.0 g/l were added to the medium. The plates were streaked and scored for reaction 48 h after incubation in darkness at 25°C.

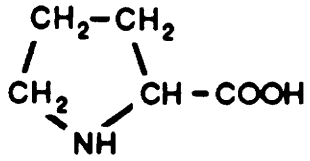
Initially, another medium for inducing fluorescence (S) (64) was supplemented with 10 mg/l crystal violet, then



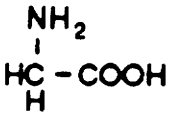
Arginine



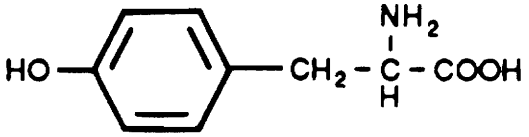
Histidine



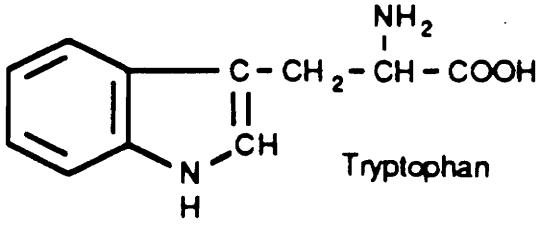
Proline



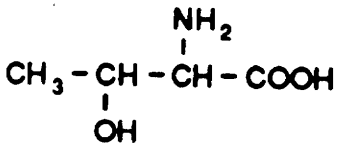
Glycine



Tyrosine



Tryptophan



Threonine

Fig. 5. Chemical structure of amino acids used as alternate nitrogen sources in PF medium.

Table 3. Succinate medium (S) for the isolation of fluorescent pigments from fluorescent pseudomonads.

Component	g/l
K_2HPO_4	6.0
KH_2PO_4	3.0
$(NH_4)_2SO_4$	1.0
$MgSO_4 7H_2O$	0.2
Succinic Acid	4.0
pH 7.2 adjusted with 2N NaOH	

streaked with M-17 and observed for reaction 48 h after incubation in the dark at 25°C (Table 3). It should be noted that the medium contained only inorganic nitrogen and used succinic acid as the carbon source.

To determine if the carbon source had an effect on the reaction, several alternate carbon and nitrogen sources were substituted into both the PF and S media and the reaction evaluated as described above. For example, succinic acid was substituted for glucose in the PF medium and either asparagine or ammonia were used as the sole nitrogen sources. Additionally, glucose was substituted for succinic acid in the S medium and either asparagine or potassium nitrate were used as the nitrogen source. All variations of the S medium were tested at two pH levels (5.5,7.0). The plates were streaked, incubated and observed as above.

4. pH effects

To determine if the color change was strictly due to a change in pH, 1.0 M HCl or 1.0 M NaOH was added dropwise to a 10 mg/l CV solution. The pH and any color changes were noted. To determine whether the initial pH of the medium had an effect on the decolorization reaction, the PF medium was adjusted to pH levels 3-8 prior to autoclaving, streaked

with M-17, and scored for reaction 48 h after incubation in darkness at 25°C.

5. Temperature effects

To determine if the incubation temperature has an effect on the reaction, cultures were streaked onto PF medium containing 10 mg/l C.V. and incubated in darkness at 14, 25, 30 or 37°C and observed daily for reaction over 21 days.

6. Agitation effects

To determine other environmental conditions which might promote or inhibit the reaction, M-17 was grown in PF medium broth containing 10 mg/l crystal violet (no agar added). The cultures were either incubated in the light at 25°C and shaken at 150 rpm, or incubated in the dark at 25°C and kept stationary for 28 days. The cultures were observed daily for reaction.

7. Reactions with similar compounds

To determine if the decolorization reaction was unique

to CV and strain M-17, several fluorescent pseudomonad strains (TR-21, BD-413, M-17) were grown on solidified PF amended with either CV, another triphenylmethane dye malachite green, (10 mg/l) or the pH indicator cresol red (10 mg/l). The cultures were incubated in darkness at 25°C and examined every 24 h for four days.

B. Identification of Crystal Violet Metabolites

As previously stated, N-demethylation was identified as the primary form of CV metabolism in diverse organisms (12,62,87). However, because M-17 is a different species and the halo formation has not been reported elsewhere, it is valuable to identify what metabolism, if any, contributes to this phenomenon. To identify the metabolites, the crystals harvested from decolorized broth cultures were considered to be the same metabolites as those in the halo regions around bacterial growth. The nature and amount of crystalline material in broth cultures facilitated their isolation without the accompanying impurities obtained from extracting large quantities of used PF agar medium. Therefore, the identification of any CV metabolites was based on this material.

1. Isolation and purification

To obtain a sufficient quantity of the decolorized precipitate (crystals) to perform an identification of metabolites, 4.0 liters of PF medium containing 30 mg/l crystal violet was dispensed into 250 ml flasks that were inoculated with M-17. Cultures were incubated in the dark at 30°C. When the broth had lost all purple color, the precipitate was pipetted out, and centrifuged at 3000 rpm for ten min and washed with distilled water. This procedure was repeated three times.

2. Solubility

To minimize the presence of any media components or bacterial metabolites contained in the water, the precipitate was dissolved in methylene chloride, and all water was pipetted off. The methylene chloride was evaporated in a warm water bath (30 min at 35°C) in a fume hood. The remaining precipitate was then dissolved in 2 ml methanol and the solution used for thin layer chromatography.

In addition to dissolving the precipitate for metabolite characterization, the solubility of the

precipitate was examined in a variety of polar and non-polar solvents (excess water, methanol, ethanol, chloroform, methylene chloride, xylene, benzene, and carbon tetrachloride) and the solubility and reaction noted . Other treatments included adding 1% pronase to the precipitate and incubating this mixture in the light for 24 h at either room temperature or 60°C to determine if water insolubility was a result of CV associating with a compound of bacterial origin. Sonication of this mixture was performed for 24 h to examine other conditions which the would solubilize the precipitate. Additional treatments included adding either pure Triton-X-100, or sodium dithionate crystals and 5 ml water to test tubes containing the precipitate and any reaction was noted.

3. Thin Layer Chromatography

TLC was used to separate the metabolites of CV. This was achieved by using a 90:9:1 propanol:H_2O:glacial acetic acid solvent system previously published (12). In this procedure, a 12.5 mg/ml CV in methanol solution was used as a standard. The metabolite solution is described above. A (40 μl) sample from each solution was spotted onto a silica gel (metal-backed) thin layer chromatography plate. The

Table 4. King's B medium for fluorescent pseudomonads

Compounds	g/l
Proteose peptone #3	20
Glycerol	10
K_2HPO_4	1.5
$MgSO_4$ $7H_2O$	1.5
Bacto Agar	15
pH 7.2	

chromatogram with standard and unknown was placed into a 250 ml graduated cylinder to which an appropriate amount of solvent had been added, and the solvent was allowed to rise for 14 h to achieve adequate separation of bands. The color and R_f values of each band were then recorded.

C. Mutagenesis of *P. putida* M-17

1. Chemical

In an effort to produce mutants of M-17 incapable of decolorizing CV at 10 mg/l, broth cultures of M-17 in Kings B medium (Table 4) were incubated for 24 h in the light at 25°C on an orbital shaker (150 rpm). Ethylmethane sulfonate (EMS) was then added to the cultures at 5% vol/vol as a mutagen, followed by incubation in the light at 25°C on an orbital shaker for twenty minutes. The mutagen was washed off by centrifugation, decanting the liquid and resuspending the cells in fresh medium to the original volume. A serial dilution of the cultures was made and 0.1 ml of the 10^{-3}-10^{-6} dilutions was spread onto plates of Kings B medium. These plates were incubated for 48 h in the dark and colonies were picked with sterile toothpicks and transferred onto plates of PF medium containing 10 mg/l CV. A total of 50 colonies

were transferred to each plate of PF medium, and incubated for 48 h at 25°C to allow for the decolorization reaction to occur. Colonies which did not appear to produce a halo were re-streaked twice onto PF medium containing crystal violet to allow for confirmation of mutant phenotype (cry-). An alternative procedure to induce mutations was to add nitrosoguanidine (2mg/ml) as a mutagen to broth cultures as above and incubate the cultures for 4 h at 30°C (65). The selection for mutants was identical to the procedure above.

2. Transposon

An alternative method of producing mutants of M-17 unable to decolorize crystal violet is the use of a transposable genetic element. The procedure used was a triparental mating with E. coli pBR322::Tn 5 as a donor of the transposable element Tn 5, E. coli pRK2013 as a helper and P. putida M-17 as the recipient strain. Cultures of each bacterium were grown to exponential growth phase in tryptic soy broth (TSB) (Table 5) and adjusted to optical density (O.D.) 1.0 at 550 nm. Portions (0.1 ml) of each culture were drawn into one sterile syringe and passed through a membrane filter (0.5 μm pore size) that trapped the bacteria. The filter was aseptically transferred to a

Table 5. Tryptic soy agar medium.*

compounds	g/l
Trypticase soy agar	4.0
Bacto-agar	15
pH 5.5	

* TSB is prepared without the agar.

general growth medium (TSB) and incubated in the dark at 28°C for 24-48 h. Cells were washed off the filter by vibrating the filter in 1 ml of sterile distilled water in a 1.5 ml Epppendorf tube. A serial dilution was made and 0.1 ml of each dilution (10^{0}-10^{-5}) was spread on plates of Davis medium supplemented with 100 mg/l kanamycin (Table 6). This permitted growth of only pseudomonad cells transformed with the Tn5 element that contained a kanamycin resistance gene. Plates were incubated at 28°C in the dark until colonies became visible. Colonies were transferred onto PF medium containing 10 mg/l CV and screened for mutant phenotypes as previously described.

D. Biological Disease Control

Cultures of strain M-17 and five cry- mutants of M-17 were grown in 250 ml flasks containing 100 ml of TSB broth on an orbital shaker (150 rpm) for 24 h at 25° C. Twenty seeds of Gossypium hirsutum L.(cotton) cv. Deltapine 50 were sown in 60 x 30 cm galvinized aluminum trays containing a 10 cm deep layer of a 1:1 sand:vermiculite mix that was previously infested with the pathogens R. solani and P. ultimum. The treatments consisted of: (1) the control; (2) the fungicide treatment [Terrachlor Super X

Table 6. Davis medium.

components	g/l
Glucose	1.0
K_2HPO_4	7.0
KH_2PO_4	2.0
$MgSO_4$ $7H_2O$	0.1
Sodium citrate pentahydrate	0.5
$(NH_4)_2SO_4$	1.0
Noble agar	15.0
Kanamycin	0.3

(Uniroyal) applied in furrow at 11.2 kg/ha]; (3) the bio-control agent M-17 applied in furrow at 1 ml/seed or 11.2 kg/ha; (4-8) and 5 cry- mutants of M-17 applied at the same rate as M-17. Each treatment was replicated four times. The trays were placed randomly in the greenhouse and watered as needed. Supplemental lights were provided by sodium vapor lamps on a 12 h photoperiod and temperatures were maintained at 28°C daytime and 23°C at night. Evaluations of stand counts were recorded 28 days after planting.

Chapter IV

RESULTS AND DISCUSSION

A. Cultural Influences on Crystal Violet Decolorization.

1. Inducibility

The experiment to determine if the decolorization reaction was constituitively produced on Kings B and PF media demonstrated that sections of media not containing CV and located either adjacent to, or at various distances from bacterial growth produced the decolorization reaction when transferred to fresh agar containing CV (Figs. 6,7). Sections removed further from the bacterial growth zone produced a smaller halo than sections taken closer to the growth which demonstrates that this phenomena is a result of the cometabolism. This also illustrates the diffusability and extracellular efficacy of the decolorizing agent.

2. Crystal violet as an energy source

Experiments using CV as the sole carbon or nitrogen source showed that the bacterium was unable to use CV as either a sole carbon or nitrogen source as no growth was observed under any conditions described.

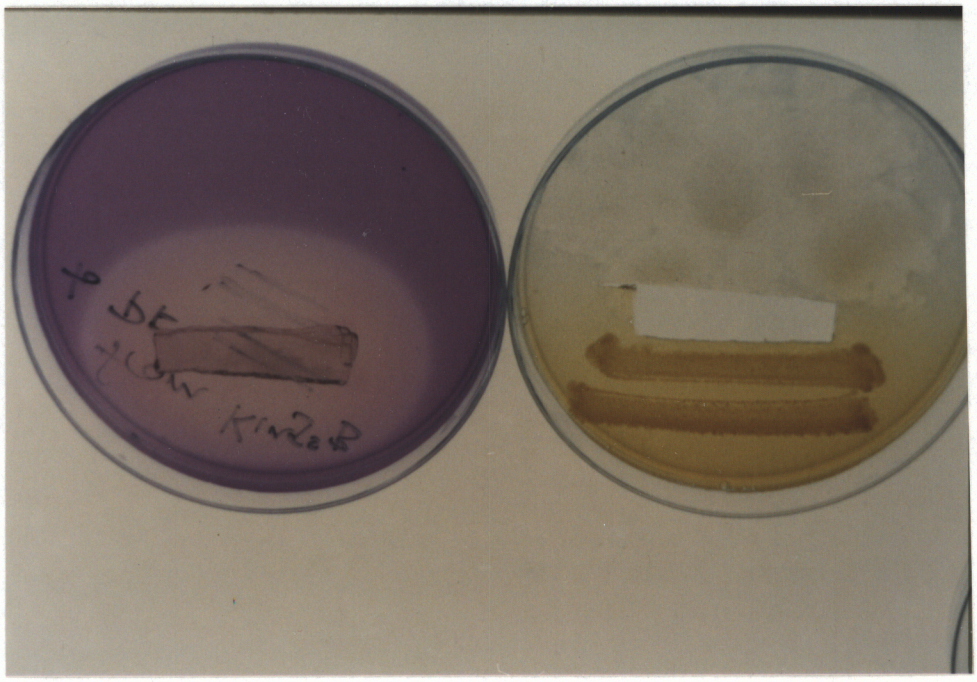


Fig. 6. Section of King's B medium cut parallel to bacterial growth of P. putida M-17 and transferred to PF medium containing crystal violet.

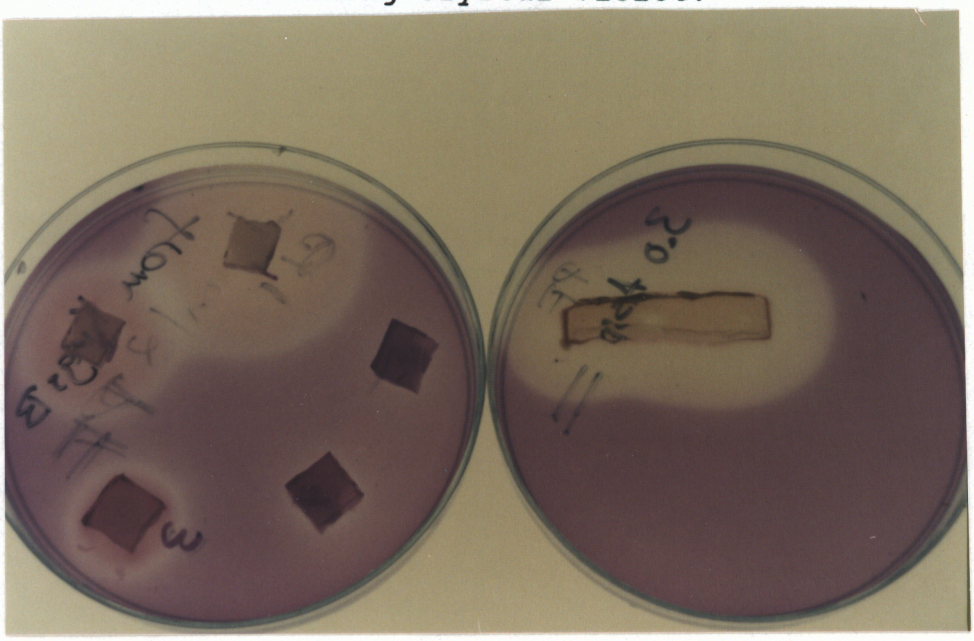


Fig. 7. Section of King's B medium without crystal violet cut either parallel or perpendicular to bacterial growth and transferred to PF medium containing CV.

3. Carbon and Nitrogen Source Effects

The intensity of the decolorized halo around a streak of M-17 was affected by the asparagine concentration when the PF medium was used. All concentrations tested produced a positive reaction, however higher concentrations of asparagine produced a lighter shade of red and had a small clear zone between the purple and red regions (Fig. 8) (Table 7).

When other amino acids were substituted for asparagine in the PF media, a variety of responses was obtained. The amino acids asparagine, arginine, glutamine, and histidine all elicited a positive reaction within 48 h (Figs. 9). Glycine, proline, and tyrosine did not elicit at 48 h, but a weak positive reaction was observed after 96 h. Aspartic acid and lysine elicited a pink/blue reaction after 96 h. Glutamic acid and tryptophan elicited a bluish halo after 96 h. Cysteine and threonine did not elicit any visible reaction after 96 h (Fig. 10, Table 8). The reaction was totally inhibited by substituting ammonia, nitrate, or urea as the sole nitrogen sources (Fig.11 and Table 8). When a

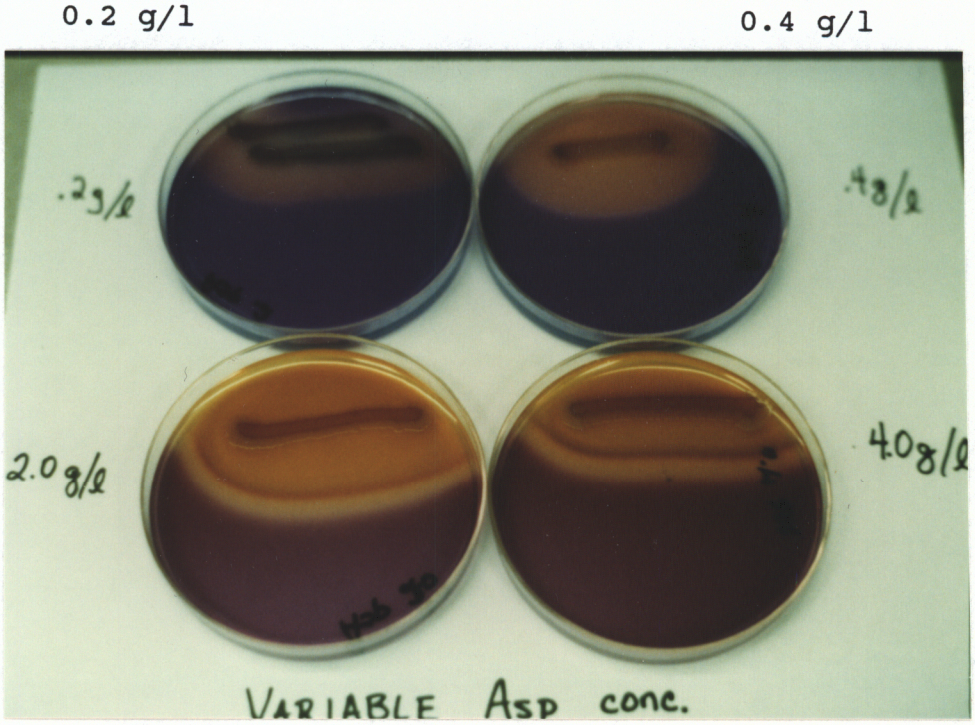


Fig. 8. Effects of variable aspargine concentrations on crystal violet metabolism in PF medium.

Table 7. Effects of additional nitrogen sources on the decolorization of crystal violet by P. putida (M-17) grown on PF medium at pH 5.5.

Asparagine concentration	Additional N	Decolorization 24 h	Decolorization 48 h
4.0 g/l		+	+
2.0 g/l		+	+
0.4 g/l		+	+
0.2 g/l		+	+
0.2 g/l	+0.1g/l $(NH_4)_2SO_4$	+	+
0.2 g/l	+1.0g/l $(NH_4)_2SO_4$	-	-
0.2 g/l	+2.0g/l $(NH_4)_2SO_4$	-	-
0.2 g/l	+0.1g/l Urea	+	+
0.2 g/l	+1.0g/l Urea	-	-
0.2 g/l	+2.0g/l Urea	-	-

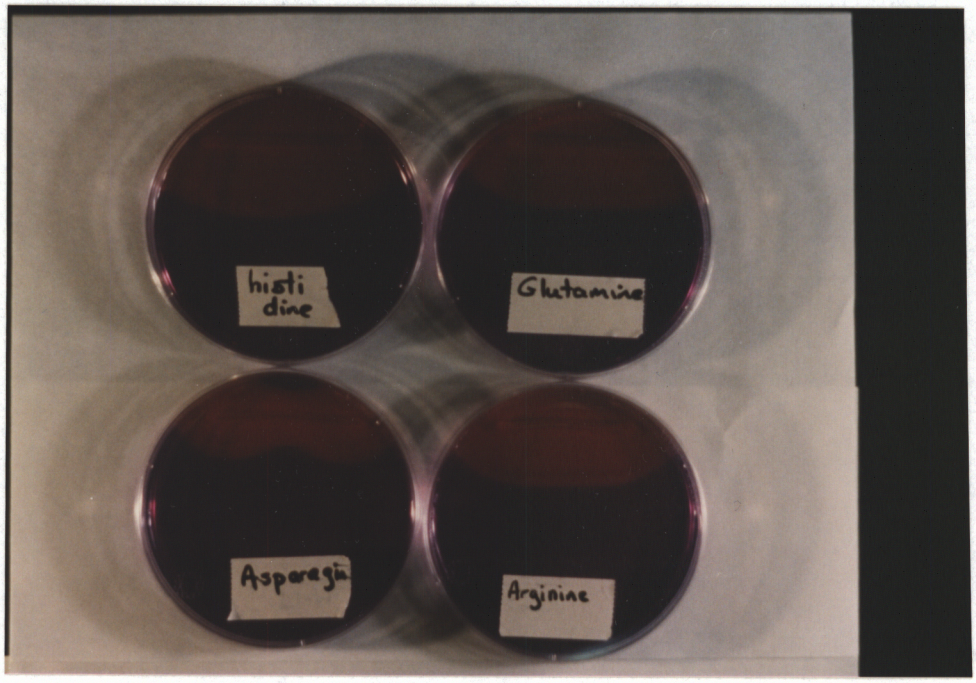


Fig. 9. Effects of alternate amino acids asparagine, glutamine, histidine and arginine on the decolorization of CV by P. putida (M-17) on PF medium.

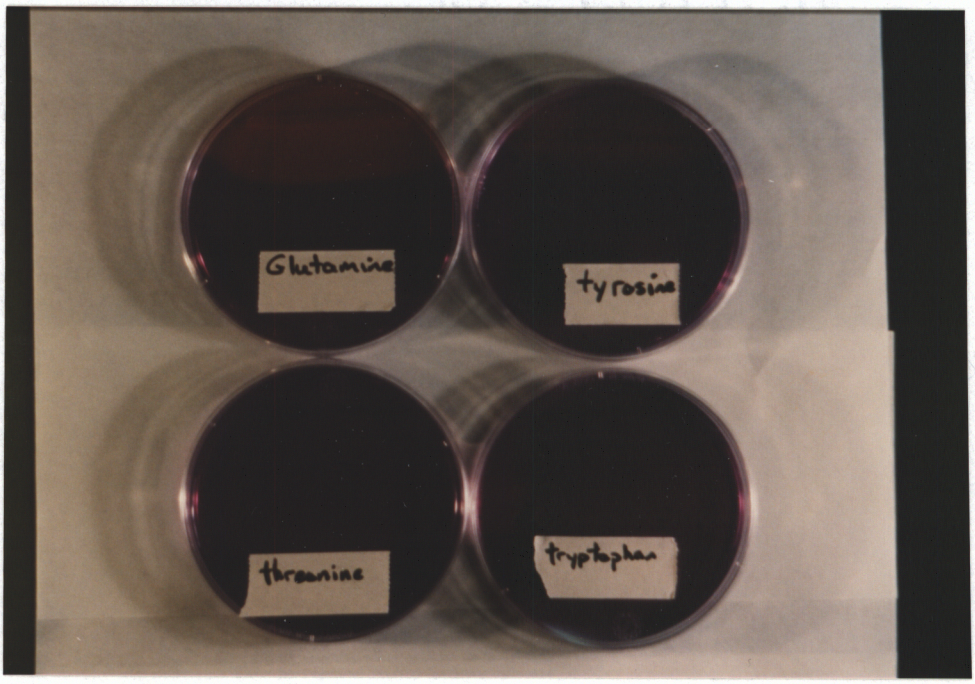


Fig. 10. Effects of amino acids glutamine, threonine, tryptophan and tyrosine on the decolorization of CV by P. putida (M-17) on PF medium.

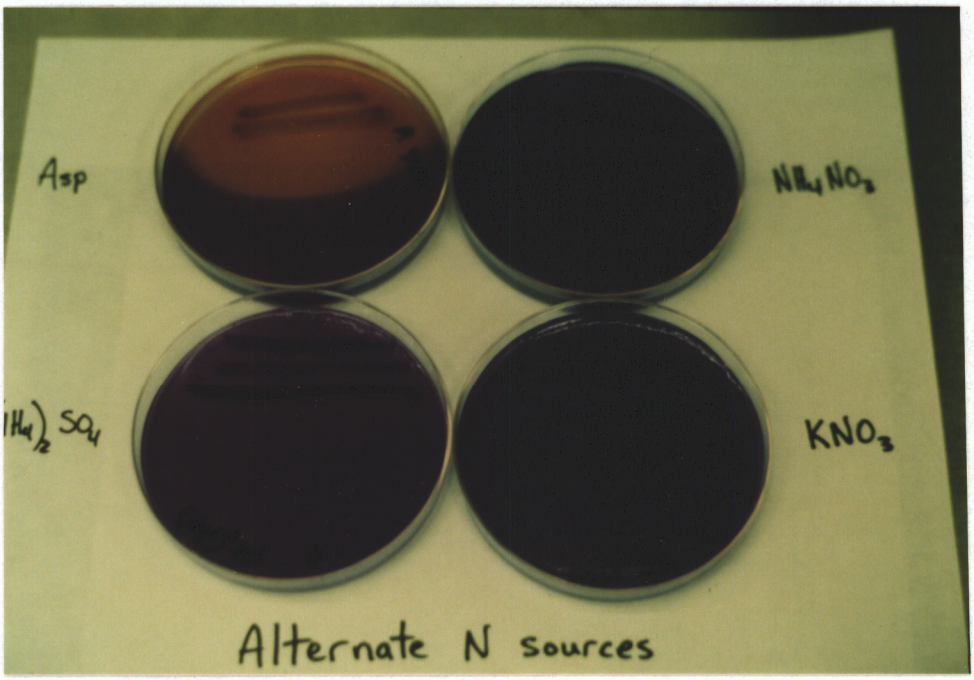


Fig. 11. Effects of alternate nitrogen sources of the decolorization of crystal violet by Pseudomonas putida (M-17) on PF medium,

Table 8. Effects of alternate nitrogen sources on the decolorization of crystal violet by P. putida (M-17) grown on PF medium at pH 5.5.

Regular Nitrogen Source	Decolorization 48 h	Decolorization 96 h
Asparagine 1.0 g/l	+	+
Alternate Nitrogen Sources *		
KNO_3	-	-
$(NH_4)_2SO_4$	-	-
NH_4NO_3	-	-
Urea	-	-
Glutamine	+	+
Aspartic acid	-	+ pink/blue
Glutamic acid	-	+ blue
Arginine	+	+
Lysine	+ weak	+ weak
Histidine	+	+
Glycine	-	+ weak
Proline	-	+ weak
Tyrosine	-	+ weak
Tryptophan	- blue	- blue
Cysteine	_	-
Threonine	_	-

* All substutitions were at a 1.0 g/l concentration.

mixture of 0.2 g/l asparagine and either ammonia or nitrate was used, the reaction was completely inhibited by the inorgainc nitrogen concentrations of 1.0 and 2.0 g/l, but did occur when inorganic nitrogen was 0.1 g/l (Fig. 12 and Table 7). These results indicate that nitrogen metabolism is a significant part of the regulation of the reaction that causes the dye to change color from purple to red to eventual clearing.

Although the determination that specific of nitrogen sources can induce the decolorization reaction could provide insight on its regulation, further experiments were conducted beyond testing ammonia, nitrate, and urea as inhibitory to the reaction. The reasons for continuing were that, on all media tested to that point, the region which was changed from purple to red also fluoresced. Secondly, any medium on which the color change did not occur also did not fluoresce. Thirdly, a paper describing the isolation of fluorescent compounds indicated that cultures should not be aerated (64). A fourth indication of the relationship between fluorescence and dye decolorization was that when the metabolites of the dye were subjected to thin layer chromotography, a region of fluorescense was observed at the solvent front. Therefore, an attempt to separate these two phenomena was made. Substitutions into the PF (Table 9) and

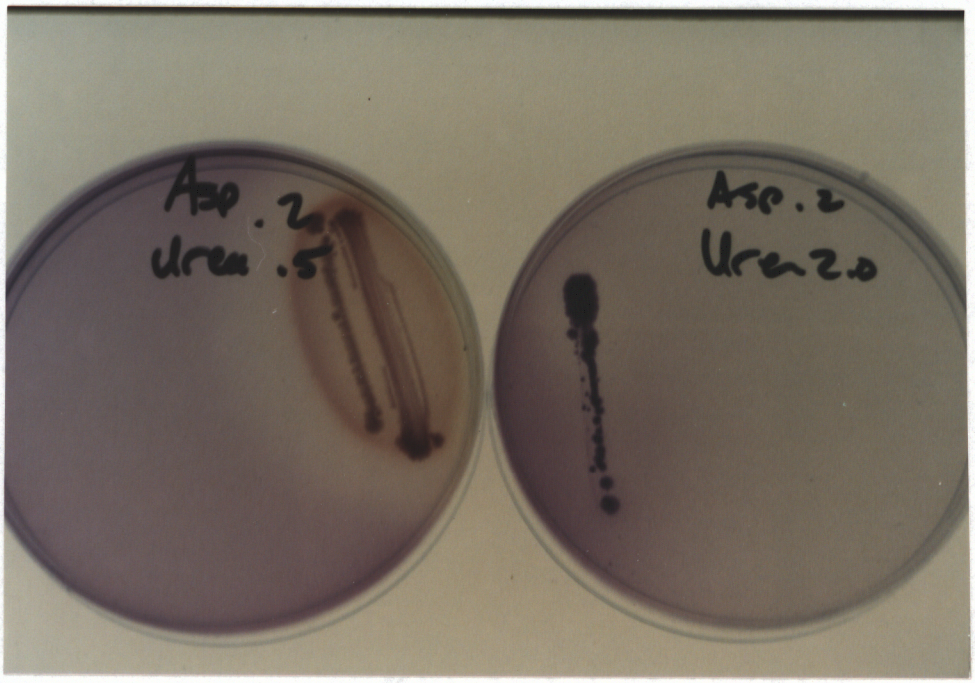


Fig. 12. Effects of urea (0.5 g/l [left] and 2.0 g/l [right]) on the decolorization of crystal violet on PF medium with reduced (0.2 g/l) Asparagine concentration.

Table 9. Effects of alternate carbon and nitrogen sources on the decolorization of crystal violet and on fluorescence by P. putida (M-17) grown on PF medium at pH 5.5 and pH 7.0.

PF media components	Glucose	Asparagine	pH	decolorization	fluorescence
Alternate Source	succinic acid	$(NH_4)_2SO_4$	5.5	+	-
	succinic acid	$(NH_4)_2SO_4$	7.0	+	+
	succinic acid	KNO_3	5.5	+ weak	+
	succinic acid	KNO_3	7.0	+ weak	+
	succinic acid		5.5	+	-
	succinic acid		7.0	not tested	

S media (Table 10) and two pH levels were used. When succinic acid was substituted for glucose in the PF medium pH 5.5 the decolorization reaction did occur and no fluorescence was observed. A similar result was noted when succinic acid was substituted for glucose and ammonia for asparagine. Therefore a separation of the two phenomena (decolorization and fluorescence) was achieved. The separation of these two phenomena would appear to conclude that they are not related, however, I am not totally convinced. Ammendments to the PF medium should be noted as this appears contrary to previous experiments in which only specific forms of amino acid nitrogen induced the reaction and ammoniacal forms of nitrogen inhibited the reaction. However, it should be noted that succinic acid can be metabolized to glutamic acid through α-ketoglutamic acid, and that glutamic acid was one of the nitrogen source substitutions in the PF medium that produced a positive reaction. Therefore the significance of a positive reaction using succinic acid and ammonia should be minimized. Therefore, these experiments, while not conclusive in identifying a specific nutritional source as critical to the regulation of this reaction, do show that the reaction is inducible, not by crystal violet, but by the nutritional sources in the medium.

Table 10. Effects of substitutions in succinic acid medium of fluorescence and crystal violet metabolism by Psuedomonas putida (M-17).

S medium components	Succinic Acid	$(NH_4)_2SO_4$	pH	Decolorization	Fluorescence
Alt. components	Glucose	Asparagine	7.0	+	+
	Glucose	Asparagine	5.5	+ weak	+ weak
		Asapargine	7.0	+	+
		Asparagine	5.5	+ weak	+ weak
	Glucose		7.0	-	-
	Glucose		5.5	-	-
		KNO_3	7.0	+ weak	-
		KNO_3	5.5	-	-
	Succinate		7.0	+	+
	Succinate		5.5	+ weak	+

4. Reaction pH and temperature dependance

To determine if the reaction was a result of localized pH change, acid was added to a crystal violet solution and no color change was observed until pH reached 1.0 when the solution changed to yellow. No effect was observed when a solution of crystal violet was made basic to pH 10. Similarly the color reaction occurred in the presence of the bacterium on PF media adjusted to pH 3-8, but was most intense at pH 5-6 (Fig. 13-16). The decolorization reaction also took place in the dark at all temperatures where growth was observed (14, 21, 25, 30°C).

5. Agitation

Another observation related to conditions regulating decolorization, was that broth cultures of PF medium containing crystal violet would not decolorize if the cultures were continually shaken. The broth cultures of the same medium that were not shaken would decolorize the dye after a period of several weeks (Fig. 17). The

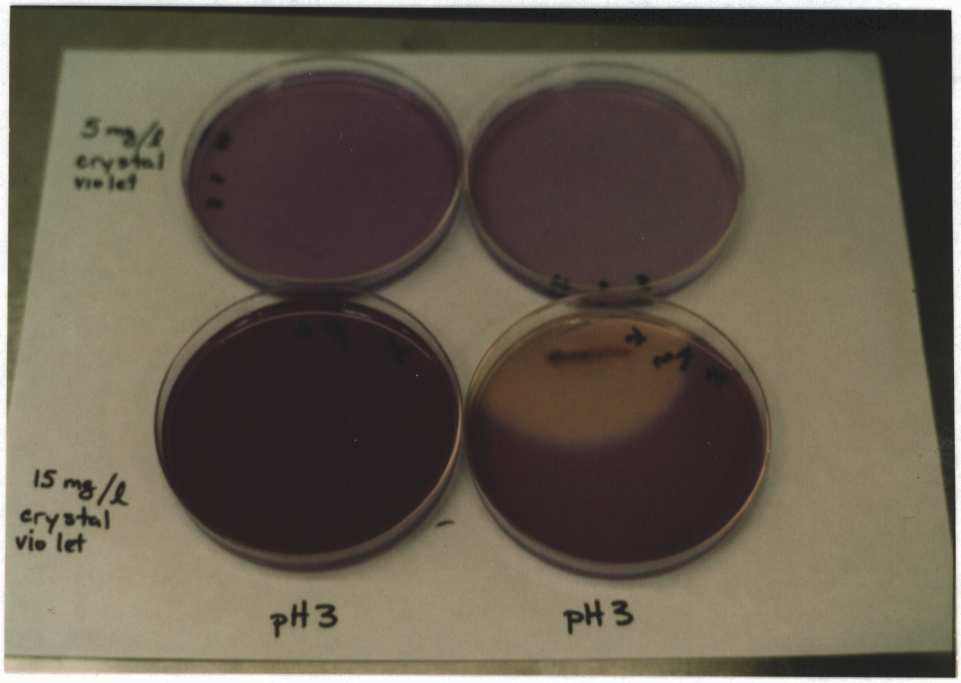


Fig. 13. Effects of pH 3.0 on the decolorization of crystal violet by Pseudomonas putida (M-17) on PF medium.

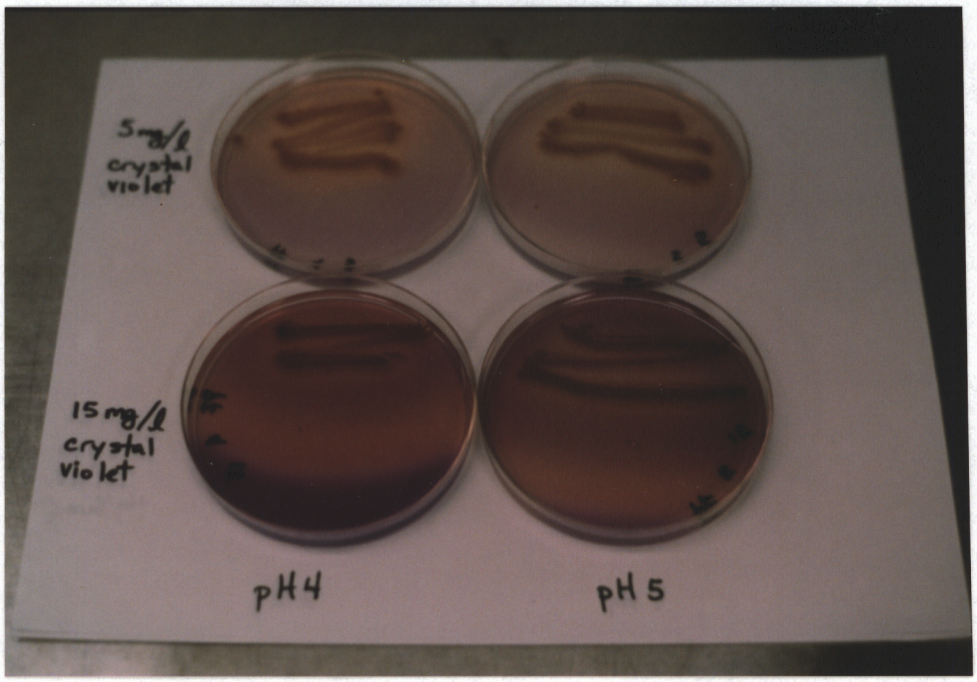


Fig. 14. Effects of pH 4.0, 5.0 on the decolorization of crystal violet by Pseudomonas putida (M-17) on PF medium.

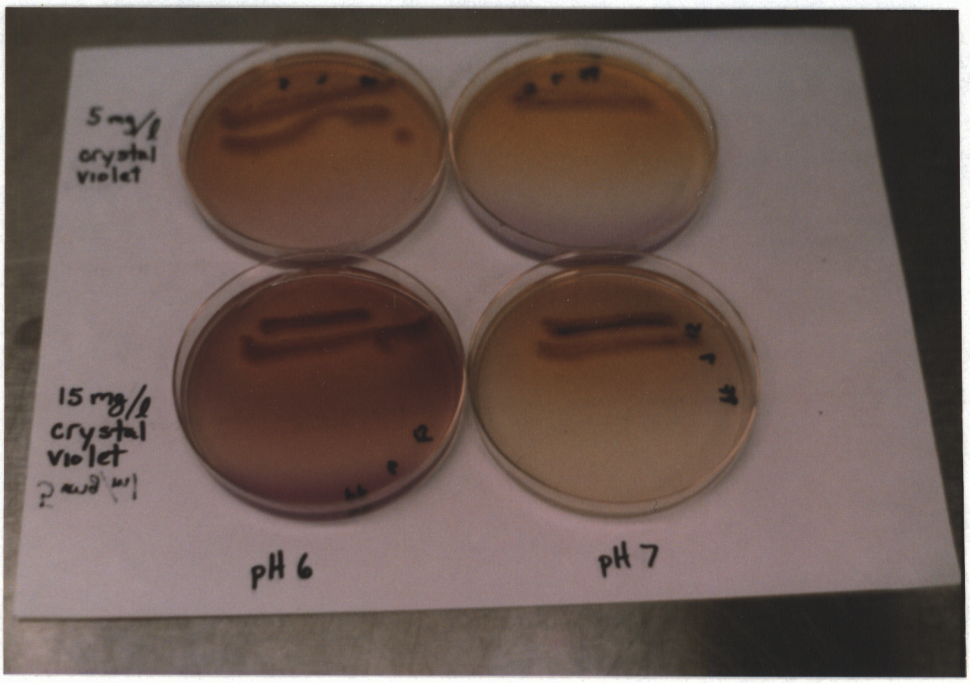


Fig. 15. Effects of pH 6.0, 7.0 on the decolorization of crystal violet by Pseudomonas putida (M-17) on PF medium.

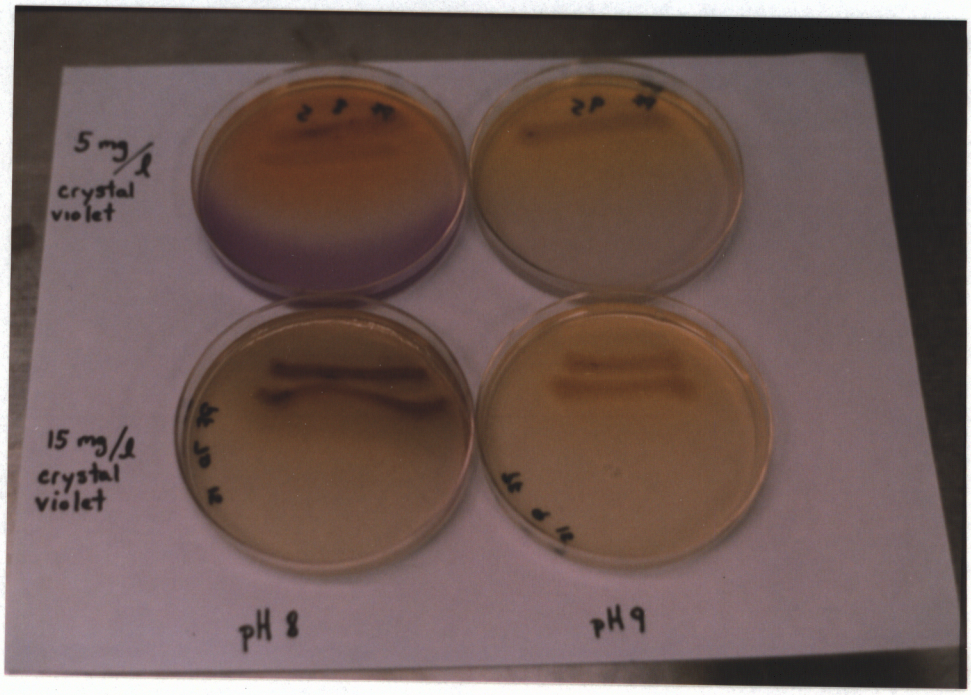


Fig. 16. Effects of pH 8.0, 9.0 on the decolorization of crystal violet by Pseudomonas putida (M-17) on PF medium.

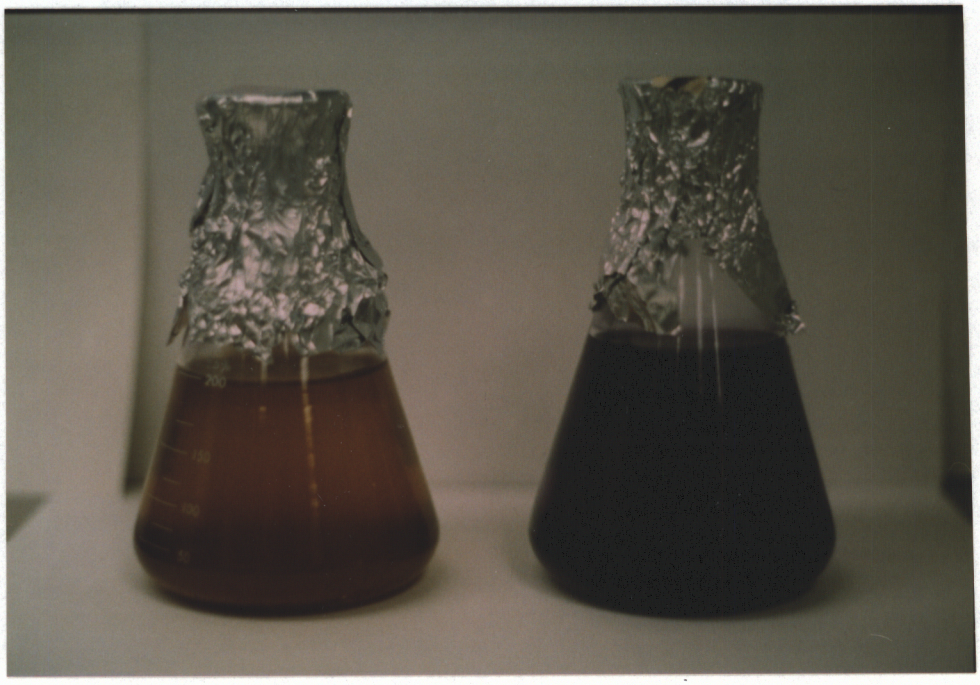


Fig. 17. Effects on the decolorization of crystal violet by P. putida (M-17) in shaken (right) and still (left) PF broth cultures containing 30 mg/l crystal violet.

decolorization reaction started from the bottom of the flask and proceeded toward the top, eventually decolorizing the contents of the entire flask. At this point a pink-maroon colored precipitate was observed at the bottom of the flask. The precipitate was examined microscopically and appeared to be crystalline and was larger than the bacterium (Fig. 18).

6. Reaction with similar compounds

The decolorization reaction of CV on PF medium containing 10 mg/l CV was unique to M-17. Two other strains (BD413, TR21) grew colonies that were a darker purple than the surrounding medium, suggesting a bioconcentration of crystal violet that did not affect their growth. The decolorization reaction appears to be specific to crystal violet, as all three strains, when grown on PF medium containing 10 mg/l malachite green produced a region around their colonies of a lighter green; but no decolorization of the dye was observed. When cresol red was added to the PF medium the strains induced a color change from yellowish to red, and was determined to be a localized pH change due to bacterial metabolism. A measurement of the pH localized to the region of color change in the media gave values of pH 8.0-8.1.

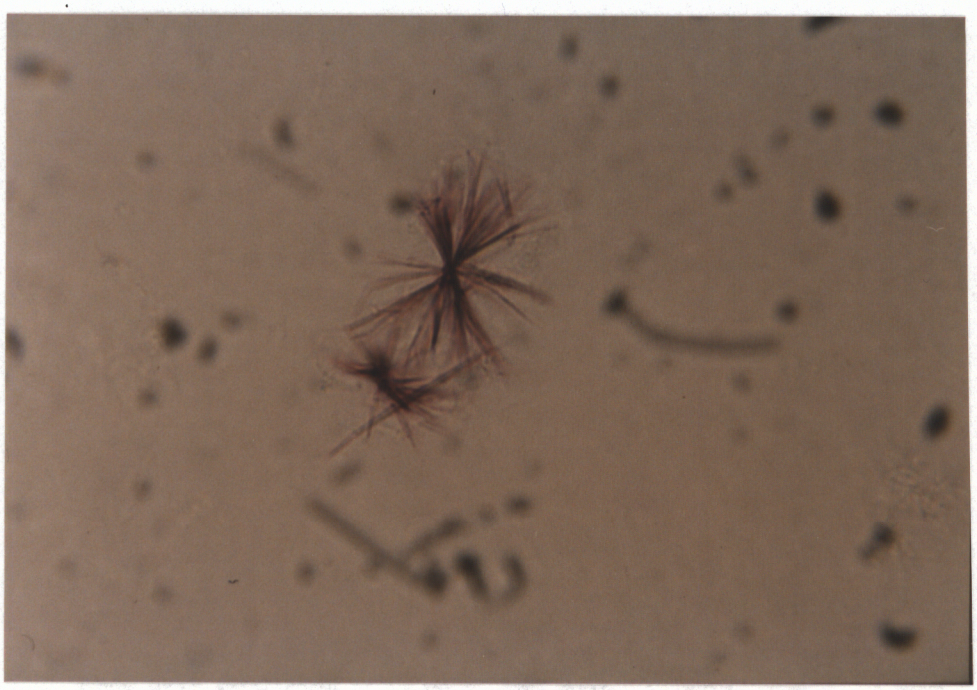


Fig. 18. Material harvested from decolorized unshaken broth cultures of P. putida (M-17) grown in PF medium containing 30 mg/l crystal violet, mag. 400x.

B. Identification of Crystal Violet Metabolites

1. Solubility

The following observations were made concerning the solubility of the precipitate. The precipitate was insoluble in water. Sonicating the precipitate in water had no visible effect. Pronase (1%) for 24 h at either 25° C or 60° C had no effect. Attempts to dissolve the precipitate in non-polar organic solvents resulted in the precipitate remaining insoluble and retaining its color in varying shades of pink-maroon colors. When treated with polar organic solvents, the precipitate dissolved and the solution was usually purple or bluish-purple (Table 11).

2. Thin layer chromotography

Thin layer chromatography was used to determine the identity of the unknown CV metabolites, as a TLC solvent system to resolve various demethylated forms of CV (the usual type of CV metabolism) was recently published (12). To determine whether CV had been demethylated by M-17, a methanol solution containing the unknown metabolites was

spotted on a TLC plate with a crystal violet standard and run for 14 h to allow for sufficient separation of bands. The crystal violet standard gave two bands with R_f values at 0.25 and 0.32 designated band I and II respectively. This is in agreement with previously published values for crystal violet (12) hexamethylpararosaniline and pentamethyl pararosaniline. The metabolite yielded six bands when observed wet during the initial chromatography. These bands were designated I-VI and had R_f values of 0.25, 0.32, 0.59, 0.73, 0.82, 0.89. Band I was blue, band II was purple, band III was light violet, band IV was pink, band V was blue-purple, and band VI was faint pink.

Although not in perfect correlation with the R_f values for various demethylated forms reported by Bumpus (12), when the color of the bands are included, it appears that these bands indicate the identification of the unknown metabolite as crystal violet and its various demethylated forms (penta-, tetra-, tri-, di-, and methylpararosaniline). It was presumed that the crystalline metabolites obtained from the PF broth culture were identical to those observed as the red halos around bacterial colonies on solidified PF medium.

Table 11. Solubility of precipitate collected from unshaken decolorized broth culture of P. putida (M-17) grown in liquid PF medium containing 30 mg/l crystal violet.

Solvent	Reaction	Color
excess H_2O	insoluble	maroon
C_2H_5OH	soluble	purple
CH_3OH	soluble	purple
$CHCl_3$	soluble	blue-purple
CH_2Cl_2	soluble	blue-purple
CCl_4	insoluble	pink
xylene	insoluble	pink
benzene	insoluble	pink

One additional observation was the presence of a yellow color observed at the solvent front of the CV metabolite spot. Whether this is an artifact of the extraction and chromatography procedures, or represents crystal violet (yellow at pH<2), or is a compound that is associated with the precipitate, or is pararosaniline was not determined.

C. Mutagenesis

The majority of efforts to obtain M-17 mutants deficient in halo production was through chemical mutagenesis. Five EMS induced mutants have been identified as being unable to produce red halos around colonies. Instead of the red halo surrounding a culture streak of the mutants, there was a clear, colorless region that was slightly fluorescent (Fig. 19). The purple color from crystal violet was not observed in this area, but only outside the immediate region. Whether these mutants are still demethylating the dye or whether some bacterial agent which complexes with one of the various demethylated pararosanilines is missing was not examined. Cry- mutants were not obtained from Ntg or Tn5 mutagenesis experiments due to insufficient number of colonies observed.

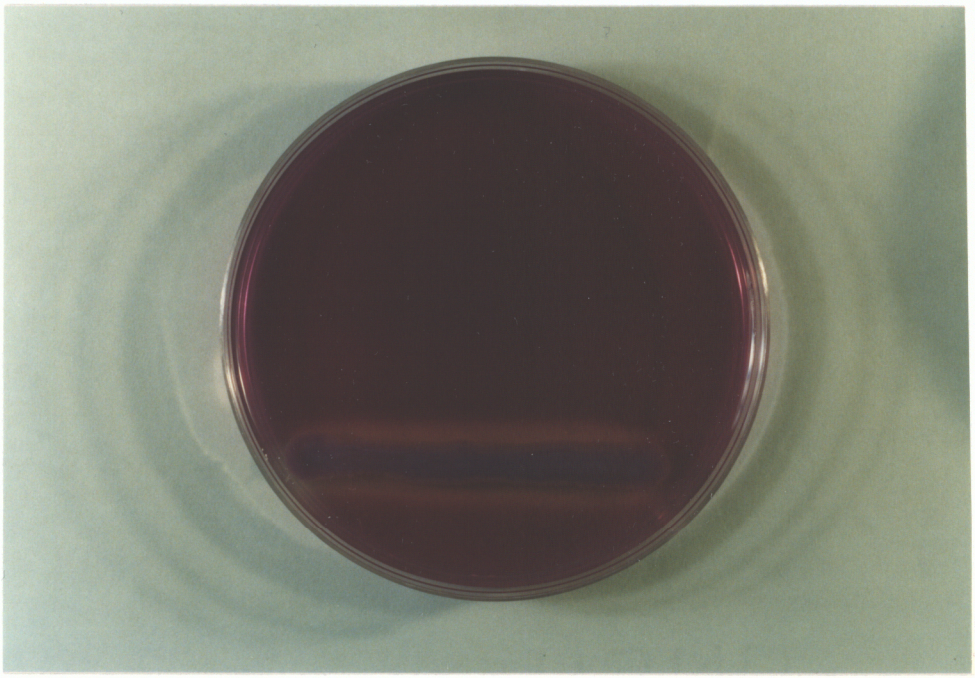


Fig. 19. Chemically induced mutant (cry-) of Pseudomonas putida (M-17) deficient in producing the maroon/pink decolorization of crystal violet when grown on PF medium containing 30 mg/l crystal violet.

D. Biological Disease Control

As part of the characterization of M-17, a greenhouse assay of its biological control capabilities was performed. Other treatments included untreated and fungicide controls and five cry- mutants of M-17. Stand counts indicated that M-17 was significantly better than the untreated control and not significantly different than the fungicide treatment. The five cry- mutants were not significantly different than either M-17 or the fungicide treatment. Three cry- mutants (2,3,5) were significantly better than the untreated control while two remaining cry- mutants (1,4) were not significantly different than the untreated control (Fig. 20, Table 12).

Table 12. Percent stand of Gossypium hirsutum cv. Deltapine 50 as a result of P. putida (M-17) or cry- mutants of M-17 innoculation on soil infested with Pythium ultimum and Rhizoctonia solani.

Treatment	Percent stand*
Untreated	38 c
Terrachlor SX**	70 ab
M-17***	67 ab
mutant #1	45 bc
mutant #2	70 ab
mutant #3	78 a
mutant #4	53 abc
mutant #5	67 ab

* All numbers followed by a letter in common were different at $p < 0.05$ level Duncan's Multiple Range Test.

** Terrachlor SX was applied at the recommended rate of 11.2 kg/ha.

*** The bacterial controls were applied at the rate of 11.2 kg/ha.

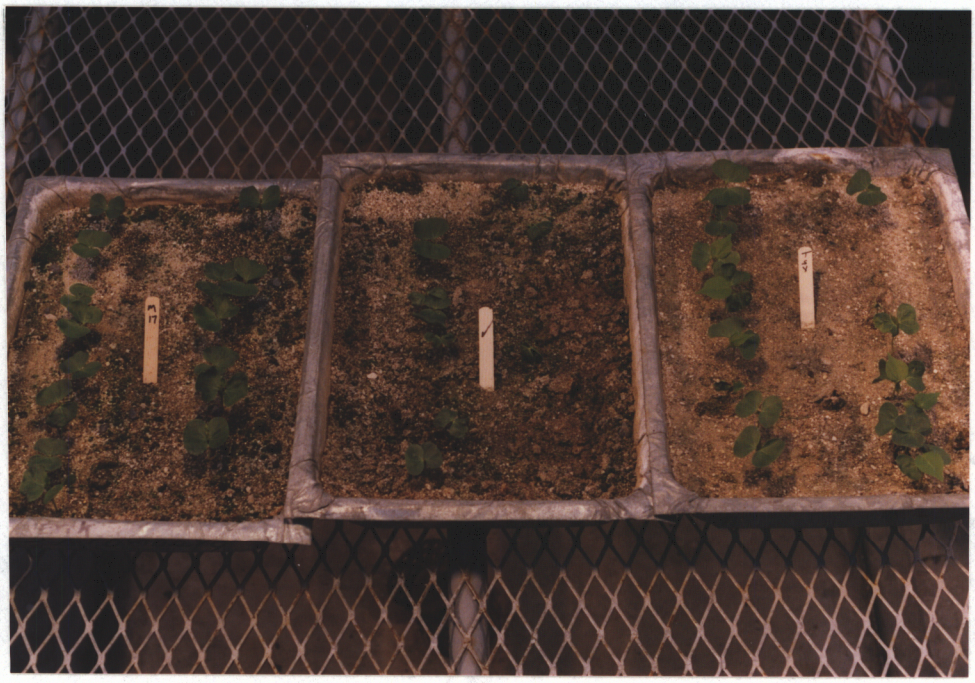


Fig. 20. Biological disease control by P. putida M-17 (left) and 1 M-17 cry- mutant of Pythium ultimum and Rhizoctonia solani on Gossypium hirsutum when compared to an untreated check (center) and Terrachlor Super-X fungicide control (right).

Chapter V

SUMMARY AND CONCLUSIONS

Fluorescent pseudomonads are highly efficient root surface colonizers with some strains capable of inhibiting several major soil-borne plant pathogens through the production of siderophore, antibiotics, and nutrient competition. In order to extend the limits and range of biological disease control exhibited by these organisms, molecular biology techniques have been employed to genetically develop superior strains and permit the study of colonization and fate of the organism in the rhizosphere and soil.

Due to environmental safety concerns, a highly selectable phenotypic trait that confers no apparent competitive advantage to the recipient organism would be a useful tool in tracking genetically engineered bacteria. One such trait has been identified as the ability of a bacterial colony to decolorize the dye crystal violet and thereby produce a red to transparent halo around the colony against a purple background. This trait was identified in P. putida M-17 by screening approximately 5000 Pseudomonas strains for unique properties. Although there are other reports for the metabolism of crystal violet, none report the formation of a

halo on solidified medium. In order to develop a useful tool from this phenomena, it would be useful to understand: (a)the mechanism of reaction; (b) how this reaction is environmentally (nutritionally) controlled; (c) the genetic element(s) required for the reaction; and (d) the effect of decolorization on biological control properties possessed by M-17. Present studies of this trait indicate the following:

1. The occurance of the red to transparent halo is not due to either pH or temperature effects,
2. The occurance of the reaction is not induced by the presence of crystal violet,
3. The reaction is constituitively produced on PF, S, TSB, and Kings B media,
4. The causal agent is water soluble, diffusable and found extracellularly,
5. The reaction in the PF medium may be inhibited by non-structurally similar forms of nitrogen (NH_4^+ , NO_3^- , urea, dissimilar amino acids),
6. The decolorization of M-17 broth cultures of PF medium containing crystal violet is inhibited by aeration,

7. A crystalline metabolite can be recovered from stationary broth cultures of M-17 in PF medium containing crystal violet,
8. The decolorization appears to be specific to crystal violet, (as a structurally similar dye, malachite green) was not decolorized by M-17,
9. The crystalline metabolite is soluble primarily in polar organic solvents,
10. The metabolite appear to be the various N-demethylated forms of crystal violet,
11. Chemically induced M-17 mutants deficient in their ability to produce the maroon halo (cry-) were identified,
12. Based on greenhouse tests, these mutants were not significantly different than the wild type M-17 in their biological control capabilities.

To continue this project, a gene library of wild type M-17 DNA needs to be constructed, then used to complement cry- mutants. The information derived from these complementation experiments should facilitate both the understanding of the number and regulation of genes involved in this phenomenon and the development of a plasmid/construct which would confer this

trait in a recipient strain that previously did not possess the ability to decolorize crystal violet. These recipients should be easily recovered from environmental samples using established techniques and one of the media outlined here. Additional mutagenesis should include the use of transposons which should provide cry- mutants with a traceable marker. This would localize the location of the gene(s) involved and would preclude the construction of a gene library. Further efforts could also be devoted to identifying the bacterial process by which the maroon color is produced. Mechanisms that have been suggested include peroxidase or other enzymes, redox reactions, or even free radical reactions.

LITERATURE CITED

1. Albert, F.G. and A.J. Anderson. 1987. The effect of Pseudomonas putida colonization on root surface peroxidase. Plant Physiol. 85:537-541.

2. Albert, F.G., L.W. Bennett and A.J. Anderson. 1985. Peroxidase associated with the root surface of Phaseolus vulgaris. J. Bot. 64:573-578.

3. Anderson, A.J. 1980. Unique aspects of the cell surface polysaccharide of Pseudomonas phaseolicola as demonstrated by bacteriophage specificity. Can. J. Microbiol. 26:1422-1427.

4. Anderson, A.J. 1983. Isolation from root and shoot surfaces of agglutinins that show specificity for saprophytic pseudomonads. Can. J. Bot. 61:3438-3443.

5. Anderson, A.J. 1984. Differences between lipopolysaccharide compositions of plant pathogenic and saprophytic Pseudomonas species. Appl. Environ. Microbiol. 48:31-35.

6. Anderson, A.J. and C. Jasalavich. 1979. Agglutination of pseudomonad cells by plant products. Physiol. Plant Path. 15:149-159.

7. Anderson, A.J. and D. Guerra. 1985. Responses of bean to root colonization with Pseudomonas putida in a hydroponic system. Phytopathol. 75:992-995.

8. Anderson, A.J., P. Habibzedegah-Tari and C.S. Tepper. 1988. Molecular studies on the root surface agglutinin in adherence and colonization by Pseudomonas putida. Appl. Environ. Microbiol. 54:375-380.

9. Bell, A. 1984. Cotton protection practices in the USA and world, Section B: diseases, p.288-309. In R.J. Kohel and C.F. Lewis (ed.), Cotton, American Society for Agronomy Monograph no.24. Madison,WI.

10. Breed, R.S., E.G. Murray, and N.R. Smith. 1957. Bergey's Manual of Determinative Bacteriology. 7 ed. Williams and Wilkins Co. Baltimore, MD. 1094pp.

11. Brindley, C., Geshcher, A., Langdon, S.P., Broggini, M., Columbo, T., and D'Incalci, M. 1982. Studies of the mode of action of antitumour triazenes and triazines-III. Metabolism studies on hexamethylmelamine. Biochem. Pharmacol. 31:625-631.

12. Bumpus, J.A. and B.J. Brock. 1988. Biodegradation of Crystal Violet by the White Rat Fungus Phanerochaete chrysosporum. Appl. Environ. Microbiol. 54:1143-1150.

13. Buyer, J.S. and J. Leong. 1986. Iron Transport-mediated Antagonism between Plant Growth-promoting and Plant-deleterious Pseudomonas Strains. Journal of Biol. Chem. 261:791-794.

14. Chao, W.L., E.B. Nelson, G.E. Harman and H.C. Hoch. 1986. Colonization of the rhizosphere by biological control agents applied to seeds. Phytopathol. 76:60-65.

15. Chen, W., H.A.J. Hoitink, A.F. Schmitthenner and O.H. Tuovinen. 1988. The Role of Microbial Activity in Suppression of Damping-Off Caused by Pythium ultimum. Phytopathol. 78:314-322.

16. Colyer, P.D. and M.S. Mount. 1984. Bacterization of Potatoes with Pseudomonas putida and its Influence on Postharvest Soft Rot Diseases. Plant Disease 68:703-706.

17. Cook, R.J. 1984. Biological control of plant pathogens: theory to application. Phytopathol. 75:25-29.

18. Cook, R.J. and D.A. Rovira. 1976. The role of bacteria in the biological control of Gaeumannomyces graminis by suppressive soils. Soil Biol. Biochem. 8:269-273.

19. Cook, R.J. and R.W. Scheider, ed. 1982. Suppressive soils and plant disease. The Amer. Phytopath. Soc. 88 pp.

20. Cook, R.J., H.T. Wilkinson and J.R. Addlredge. 1985. Evidence that microorganisms in suppressive soil associated with wheat take-all decline do not limit the number of lesions produced by Gaemannomyces graminis var. tritici. Phytopathol. 76:342-345.

21. Daniels, M.J., C.E. Barber, P.C. Turner, M.K. Sawczye, W. Byrde and A.H. Fielding. 1984. Cloning of genes involved in pathogenicity of Xanthomonas campestris pv. campestris using the broad host range cosmid pLAFR1. Embo J. 3:3323-3328.

22. Davison, J. 1988. Plant Beneficial Bacteria. Bio/ Technol. 6:282-286.

23. Dazzo, F.B., F.L. Truchet, J.E. Sherwood, E.M. Hrakab, and S.H. Pakratz. 1984. Specific phases of root hair attachment in the Rhizobium trifolii clover symbiosis. Appl. Environ. Microbiol. 48:1140-1150.

24. De Weger, L.A., I.M. Cornelia, V.D. Vlugt, A.H.M. Wifjfes, P.A.H.M. Bakker, B. Schipers, and B. Lugtenberg. 1987. Flagella of a plant-growth-stimulating Pseudomonas fluorescens strain are required for colonization of potato roots. J. Bacteriol. 169:2769-2773.

25. De Weger, L.A., Van Boxtel, R., Van Der Burg, B., Gruters R.A., Geels F.P., Schippers, B., and Ben Lugtenberg. 1986. Siderophores and Outer Membrane Proteins of Antagonistic Plant Growth Stimulating Root Colonizing Pseudomonas spp. J. Bacteriol. 165:585-594.

26. Ditta,G. 1988. Tn5 Mapping of Rhizobium Nitrogen Fixation Genes Section VI: Nitrogen Metabolism, p 331-340 In A. Weissbach and H. Weissbach (ed), Methods for Plant Molecular Biology Academic Press Inc., San Diego, CA.

27 Drahos, D.J., Hemming, B.C., and McPherson, S., 1986. Tracking recombinent organisms in the environment: -galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads. Bio/Technol. 4:439-444.

28. Dupler, M. and R. Baker. 1982. Survival of Pseudomonas putida, a biological control agent, in soil. Phytopathol. 74:195-200.

29. Duvick, J.P. and L. Sequeira. 1984. Interaction of Pseudomonas solanacerarum lipopolysaccharide and extracellular polysaccharide with agglutinin from potato tubers. Appl. Environ. Microbiol. 48:192-198.

30. Elad, Y. and R. Baker. 1985. Influence of trace amounts of cations and siderophore-producing pseudomonads on clamydospore germination of Fusarium oxysporum. Phytopathol. 75:1047-1052.

31. Elad, Y. and R. Baker. 1985. The role of competition for iron and carbon in suppression of chlamydospore germination of Fusarium spp. by Pseudomonas spp. Phytopathol. 75:1053-1059.

32. Elad, Y. and I. Chet. 1986. Possible role of competition for nutrients in biocontrol of Pythium damping-off by bacteria. Phytopathol. 77:190-195.

33. Gardiol, A.E., R.I. Hollingsworth and F.B. Dazzo. 1986. Alteration of surface properties in a Tn5 mutant strain of Rhizobium trifolii 0403. J. Bacteriol.169:1161-1167.

34. Gould, W.D., C. Hagedorn, and T.R. Bardinelli. 1986. A Pseudomonas putida isolate displaying a wide range of physiological activities. Microbiol. Abstr.,N-91,p.265.

35. Gurushddaiah, S., D.M. Weller, A. Sarkar and R.J. Cook. 1986. Characterization of an antibiotic produced by a strain of Pseudomonas fluorescens inhibitory to Gaeumannomyces graminis var. tritici and Pythium spp. Antimicro. Agnts. and Chemother. 29:488-495.

36. Gutterson, N.L., T.J. Llayton, J.S. Ziegle and G.J. Warren. 1986. Molecular cloning of genetic determinants for inhibiton of fungal growth by a flourescent pseudomonad. J. Bacteriol. 165:696-703.

37. Harwood, C.S., M. Rivelli, and L.N. Ornston. 1984. Aromatic acids are Chemoattractants for Pseudomonas putida J. Bacteriol. 160:622-628.

38. Howell, C.R. and R.D. Stipanovic. 1979. Control of Rhizoctonia solani on cotton seedlings with Pseudomonas fluorescens with an antibiotic produced by the bacterium. Phytopathol. 69:480-482.

39. Howell, C.R. and R.D. Stipanovic. 1980. Suppression of Pythium ultimum-induced damping-off of cotton seedlings by Pseudomonas fluorescens and its antibiotic, Pyoluteorin. Phytopathol. 70:712-715.

40. Howie, W.J., R.J. Cook and D.M. Weller. 1987. Effects of soil matric potential and cell motility on wheat root colonization by fluorescent pseudomonads suppressive to take-all. Phytopathol. 77:286-292.

41. James, D.W., Jr., T.V. Suslow and K.E. Steinback. 1985. Relationship between rapid, firm adhesion and long-term colonization of roots by bacteria. Appl. Environ. Microbiol. 50:293-397.

42. James, D.W. jr., and N.I. Gutterson. 1986. Multiple antibiotics produced by Pseudomonas fluorescens HV37a and their differential regulation by glucose. Appl. Environ. Microbiol. 52:1183-1189.

43. Jasalavich, C.A. and A.J. Anderson. 1981. Isolation from legume tissues of an agglutinin of saprophytic pseudomonads. Can. J. Bot. 2:264-271.

44. Kloepper, J.W., J. Leong, M. Teintze and M.N. Schroth. 1980. Pseudomonas siderophores: a mechanism explaining disease-suppressive soils. Cur. Microbiol. 4:317-320.

45. Kloepper, J.W., M.N. Schroth and T.D Miller. 1980. Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. Phytopathol. 70:1078-1082.

46. Kloepper, J.W., J. Leong, M. Teintze, and M.N. Schroth. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885-886.

47. Kloepper, J.W., R. Lifshitz, M.N. Schroth. 1988. Pseudomonas Innoculants to benefit plant production. ISI Atlas of Science. p60-64.

48. Kloepper, J.W. and M.N. Schroth. 1981. Plant growth promoting rhizobacteria and plant growth under gnotobiotic conditions. Phytopathol. 71:642-644.

49. Kloepper, J.W., F.M. Scher, M. Laliberte and I. Zaleska. 1985. Measuring the spermosphere colonizing capacity (spermosphere competence) of bacterial innoculants. Can. J. Microbiol. 31:926-929.

50. Kwasniewska, K. 1985. Biodegradation of crystal violet (hexamethyl-p-rosaniline) by oxidative red yeasts. Bull. Environ. Contam. Toxicol. 34:323-330.

51. Lacy, G.H. personal communication.

52. Lagrimini, L.M., W. Burkhart, M. Moyer and S. Rothstein. 1987. Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: Molecular analysis and tissue-specific expression. Proc. Natl. Acad. Sci. 84:7542-7546.

53. Levin, M.A., R. Seidler, A.W. Borquin, J.R. Fowle III and T. Barkay. 1987. EPA developing methods to assess environmental release. Bio/Technology 5:38-45.

54. Leong, J. 1986. Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. Ann. Rev. Phytopathol. 24:187-209.

55. Lippincott, B.B., M.H. Whatley and J.A. Lippincott. 1977. Tumor induction by Agrobacterium involves attachment of the bacterium to a site on the host plane cell wall. Plant Physiol. 59:38-390.

56. Loper, J.E. 1988. Role of fluorescent siderophore production in biological control at Pythium ultimum by a Pseudomonas fluorescens strain. Phytopathol.78:166-172.

57. Loper, J.E., Haack C., and M.N. Schroth. 1985. Population dynamics of soil pseudomonads in the rhizosphere of potato (Solanum tuberosum L.). Appl. Environ. Microbiol. 49:416-422.

58. Loper, J.E., C.S. Orser, N.J. Panopoulos and M.N. Schroth. 1984. Genetic analysis of fluorescent pigment production in Pseudomonas syringae pv. syringae. Journal Gen. Microbiol. 130:1507-1515.

59. Magazin, M.D., J.C. Moores and J. Leong. 1985. Cloning of the gene coding for the outer membrane receptor protein for ferric pseudobactin, a siderophore from a plant growth-promoting Pseudomonas strain. J. Bio. Chem. 261:795-799.

60. Marugg, J.D., M. Van Spanje, W.P.M. Hoekstra, B. Schippers, and P.J. Weisbeek. 1985. Isolation and analysis of genes involved in siderophore biosynthesis in plant-growth-stimulating Pseudomonas putida WCS358. J. Bacteriol. 164:563:570.

61. McCormick, D. 1986. EPA's novel idea for a 'suicide plasmid'. Bio/Technol. 4:5-762

62. McDonald, J.J., C.R. Breeden, B.M. North and R.W. Roth. 1984. Species and strain comparison of the metabolism of gentian violet by liver microsomes. J. Agric. Food Chem. 32:596-600.

63. Mew, T.W. and A.M. Rosales. 1986. Bacterization of rice plants for control of sheath blight caused by Rhizoctonia solani. Phytopathol. 76:1260-1264.

64. Misaghi, I.J., Stowell, L.J. Grogan, R.G., and Spearman, L.C. 1982. Fungistatic activity of water-soluble fluorescent pigments of fluorescent pseudomonads. Phytopathol. 72:33-36.

65. Moores, J.C., M. Magazin, G.S. Ditta and J. Leong. 1984. Cloning of genes involved in the biosynthesis of pseudobacitn, a high-affinity iron transport agent of a plant growth-promoting Pseudomonas strain. J. Bacteriol. 157:53-58.

66. Obukowicz, M.G., F.J. Perlak, S.L. Bolten, K. Kusano-Kretzmer, E.J. Mayer and L.S. Watrud. 1987. IS50L as a non-self transposable vector used to integrate the Bacillus thuringiensis delta-endotoxin gene into the chromosome of root-colonizing pseudomonads. Gene 51:91-96.

67. Obukowicz, M.G., F.J. Perlak, K. Kusano-Kretzmer, E.J. Mayer, S.L. Bolten and L.S. Watrud. 1986. Tn5-mediated integration of the delta-endotoxin gene from Bacillus thuringiensis into the chromosome of root-colonizing pseudomonads. J. Bacteriol. 2:982-989.

68. Parke, J.L., R. Moen, A.D. Rovira and G.D. Bowen. 1986. Soil water flow affects the rhizosphere distribution of a seed-borne biological control agent, Pseudomonas fluorescens. Soil. Biol. Biochem. 18:583-588.

69. Poplawsky, A.R., Y.F. Peng, and A.H. Ellingboe. 1988. Genetics of antibiosis in bacterial strains suppressive to take-all. Phytopathol. 78:426-482.

70. Puvanasarjah, Vl, F.M.Schell, D.Gerhold and G. Stacey. 1987. Cell surface polysaccharides from Bradyrhizobium japonicum and a non-nodulating mutant. J. Bacteriol. 169:137-141.

71. Rama Raje Urs, N.V. and J.M Dunleavy. 1975. Enhancement of the bactericidal activity of a peroxidase system by phenolic compounds. Phytopathol. 65:686-690.

72. Scher, F.M. and R. Baker. 1980. Mechanism of biological control in a Fusarium-suppressive soil. Phytopathol. 70:412-417.

73. Scher, F.M. and R. Baker. 1982. Effect of Pseudomonas putida and a synthetic iron chelator on induction of soil suppressiveness to Fusarium wilt pathogens. Phytopathol. 72:1567-1573.

74. Scher, F.M., J.W. Kloepper and C.A. Singleton. 1985. Chemotaxis of fluorescent Pseudomonas spp. to soybean seed exudates in vitro and in soil. Can. J. Microbiol. 31:570-574.

75. Sneh, B., M. Dupler, Y. Elad and R. Baker. 1984. Chlamydospore germination of Fusarium oxysporum f. sp. cucumerinum as affected by fluorescent and lytic bacteria from a Fusarium-suppressive soil. Phytopath. 74:1115-1124.

76. Stone, T.B., S.R. Sims, and P.G. Marrone. 1989. Selection of Tobacco Budworm for resistance to a genetically engineered Pseudomonas fluorescens containing the -endotoxin of Bacillus thuringiensis subsp kurstaki.J. Invert. Pathol. 53:228-234.

77. Teintze, M., M.B. Hossain, C.L. Barnes, J. Leong, and D. van der Helm. 1981. Structure of ferric pseudobactin, a siderophore from a plant growth promoting Pseudomonas. Biochem. 20:6446-6457.

78. Teintze,M., and J. Leong. 1981. Structure of pseudobactin A, a second siderophore from plant growth promoting Pseudomonas B 10. Biochem. 20:6457-6462.

79. Vesper, S.J. 1987. Production of pili (fimbriae) by Pseudomonas fluorescens and correlation with attachment to corn roots. Appl. Environ. Microbiol. 53:1397-1405.

80. Vesper, S.J. and W.D. Bauer. 1986. Role of pili (fimbriae) in attachment of Bradyrhizobium japonicum to soybean roots. Appl. Environ. Microbiol. 52:134-141.

81. Wilson, L.G. and J.C. Fry. 1986. Extensin-a major cell wall glycoprotein. Plant Cell Environ. 9:239-260.

82. Weger de, L.A., R. van Boxtel, B. van der Burg, R.A. Gruters, F.P. Geels, B.Schippers and B. Lugtenberg. 1986. Siderophores and outer membrane proteins of antagonistic, plant-growth-stimulating, root-colonizing psuedomonas spp. J. Bacteriol. 165:585-594.

83. Weller, D.M. and R.J. Cook. 1983. Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. Phytopathol. 73:463-469.

84. Xu, G.W. and D.C. Gross. 1985. Selection of fluorescent pseudomonads antagonistic to Erwinia caratova and suppressive of potato seed piece decay. Phytopathol. 76:414-422.

85. Xu, G.W. and D.C. Gross. 1985. Field evaluations of the interactions among fluorescent pseudomonads, Erwinia caratovora, and potato yields. Phytopathol. 76:423-430.

86. Yatome, C., T. Ogawa and E. Idaka. 1984. Microbial degradation of dyes-decolorization rate of dyes by several strains. Sen I Gakkaishi 9:344-349.

87. Yatome, C., D. Koga and E. Idaka. 1981. Biodegradability of azo and triphenylmethane dyes by Pseudomonas pseudomallei 13NA. J. Soc. Dyers Colour. 97:166-169.

88. Yatome, C., T. Ogawa, D. Koga, and E. Idaka. 1981. Biodegradation of several dyes by the starved cells of Pseudomonas pseudomallei 13NA. Sen I Gakkaishi 37:485-488.

89. Young, D.H., W.P.C Stemmer, and L. Sequeira. 1985. Reassembly of a fimbrial hemagglutinin from Pseudomonas solanacearum after purification of the subunit by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. 50:605-610.

90. Young, D.H. and L. Sequeira. 1986. Binding of Pseudomonas solanacearum fimbriae to tobacco leaf cell walls and its inhibition by bacterial extracellular polysaccharides. Physiol. and Mol. Plant Path. 28:393-402.

VITA

The author was born in Little Rock, Arkansas and was raised from the age of three by his grandparents (Mom and Dad) in Rutherford, New Jersey. The author recieved a B.A. in Chemistry from The Kings College in Briarcliff Manor, New York and spent an enjoyable year teaching high school chemistry in Yorktown, New York. From thence came I to this institution for the purpose of pursuing an M.S. in Agronomy. After a two year hiatus at the DNA Plant Technology Corporation as a watermelon breeder, the author returned to Virginia Tech to finish the thesis.

Fred McCuistion