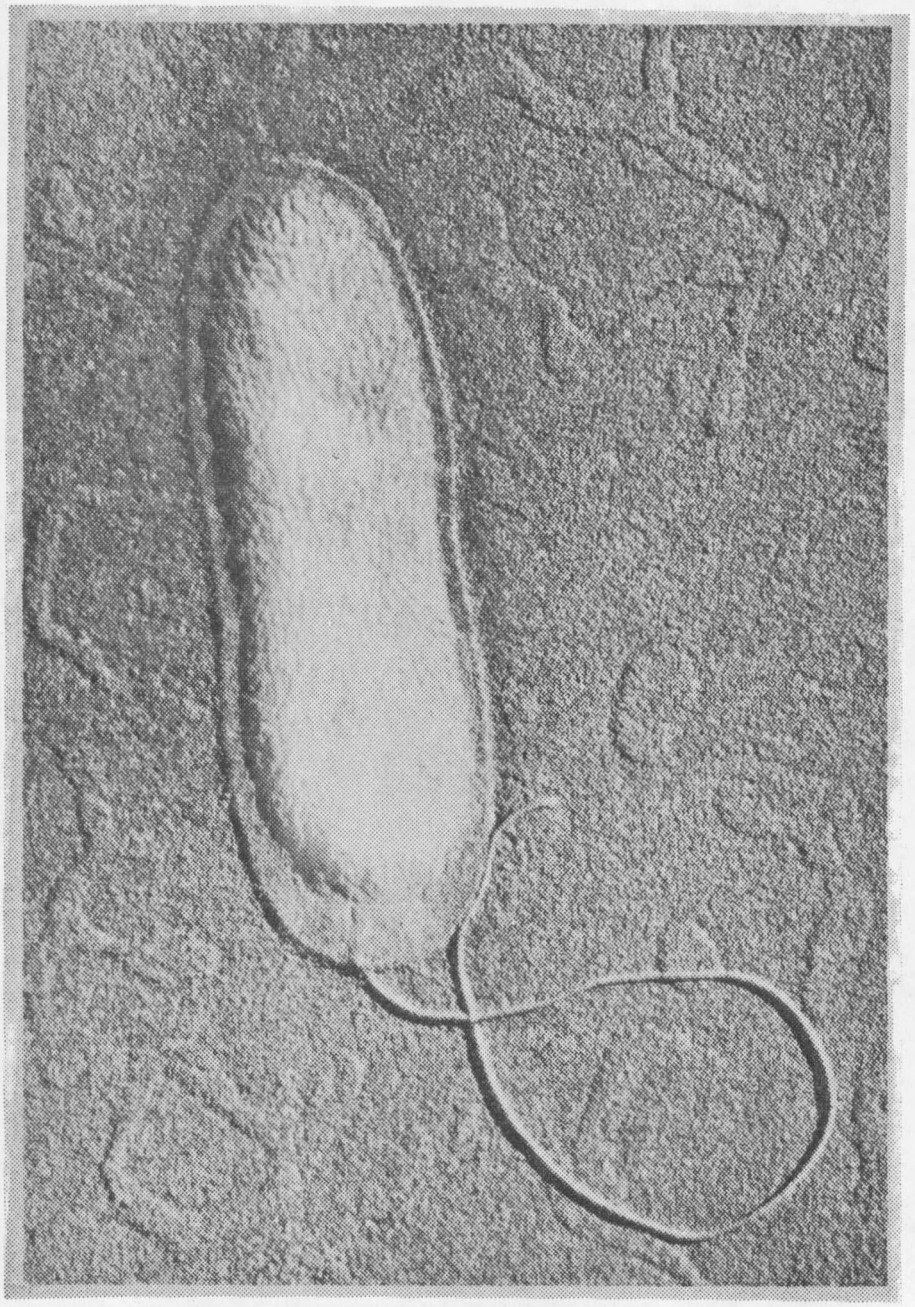
Plant-associated fluorescent pseudomonads : their systematic analysis, microbial antagonism and iron interaction

by Bruce Clark Hemming

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Plant Pathology
Montana State University

Abstract:
The plant-associated fluorescent pseudomonads are a potentially useful group of bacteria which serve as the focal point of several fields of applied research. The experiments reported represent the first systematic study of iron induced changes on microbial antagonism of these agriculturally important bacteria. A comprehensive numerical analysis which included data on 113 characters of nearly 200 isolates from a variety of plants served as the foundation on which the strain specific iron induced changes in inhibition were examined. Isolates tended to cluster on the basis of their host plant origin when characterized by carbon utilization tests. The numerical taxonomy was conducted in a manner which facilitated the evaluation of the relative merit of sole carbon source utilization tests, bacteriocin tests and antibiotic production tests in the characterization of strains. In addition, hypersensitivity response production in tobacco (Nicotiana sp.), phytopathogenicity in marigolds (Tagetes sp.), and ice nucleation ability of several isolates were determined. The ability of isolates to grow in the presence of 10.0 mM EDTA also provided data of taxonomic value. Strain variation and sectoring of colonies are discussed in terms of possible operative genetic mechanisms.

The isolation and partial characterization of an antibiotic from one isolate used in a novel treatment of Dutch elm disease revealed its ability to chelate iron and its similarity to siderophores and a phytotoxin, syringomycin. The previously unknown basic amino acid residues of syringomycin produced by Pseudomonas syringae were identified as δ -N-hydroxyornithine. Siderophore production effects both bacteriocin typing and production of fungistatic zones of inhibition by these bacteria. Antibiosis directed towards the eucaryotic organisms, Geotrichum candidum, Ceratocystis ulimi and a Rhodotorula species, increases for most pseudomonad strains on media containing iron. The results lead to the speculation that the iron status of plants and the ability of a pathogen to acquire iron may significantly affect the progression of some bacterial and fungal plant diseases and other microbial-plant interactions.



"Magnify the small but important things, since simple things can make a profound difference."

--Anonymous

(Micrograph of a pseudomonad at 40,000X magnification)

To Caroline,

Eric,

Heidi and

Heather

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ABSTRACT

The plant-associated fluorescent pseudomonads are a potentially useful group of bacteria which serve as the focal point of several fields of applied research. The experiments reported represent the first systematic study of iron induced changes on microbial antagonism of these agriculturally important bacteria. A comprehensive numerical analysis which included data on 113 characters of nearly 200 isolates from a variety of plants served as the foundation on which the strain specific iron induced changes in inhibition were examined. Isolates tended to cluster on the basis of their host plant origin when characterized by carbon utilization tests. The numerical taxonomy was conducted in a manner which facilitated the evaluation of the relative merit of sole carbon source utilization tests, bacteriocin tests and antibiotic production tests in the characterization of strains. In addition, hypersensitivity response production in tobacco (Nicotiana sp.), phytopathogenicity in marigolds (Tagetes sp.), and ice nucleation ability of several isolates were determined. The ability of isolates to grow in the presence of 10.0 mM EDTA also provided data of taxonomic value. Strain variation and sectoring of colonies are discussed in terms of possible operative genetic mechanisms.

The isolation and partial characterization of an antibiotic from one isolate used in a novel treatment of Dutch elm disease revealed its ability to chelate iron and its similarity to siderophores and a phytotoxin, syringomycin. The previously unknown basic amino acid residues of syringomycin produced by Pseudomonas syringae were identified as δ-N-hydroxyornithine. Siderophore production effects both bacteriocin typing and production of fungistatic zones of inhibition by these bacteria. Antibiosis directed towards the eucaryotic organisms, Geotrichum candidum, Ceratocystis ulmi and a Rhodotorula species, increases for most pseudomonad strains on media containing iron. The results lead to the speculation that the iron status of plants and the ability of a pathogen to acquire iron may significantly affect the progression of some bacterial and fungal plant diseases and other microbial-plant interactions.

INTRODUCTION

Members of the bacterial genus *Pseudomonas* are common inhabitants of plant, soil, fresh and marine water environments, often as one of the dominant members of the microflora. This may be, in part, a result of their capability of growth in a purely mineral medium with the addition of an appropriate, simple carbon source. These Gram-negative, aerobic, motile (by means of polar flagella) bacteria exhibit a respiratory metabolism and are never fermentative. Diffusable fluorescent pigments are produced by some species, most notably in iron-deficient media. Colonies of "fluorescent" pseudomonads are characterized by yellow-green fluorescence when viewed under ultra-violet light. The fluorescent pseudomonads form a verified, related group of species within the genus, *Pseudomonas* (24). Species within this group and their associations with plants and soils are seemingly ubiquitous.

Saprophytic soil and plant epiphytic strains are found in the fluorescent pseudomonad group, as well as, the preponderate number of phytopathogenic strains of the genus. Of the major demonstrated plant pathogenic pseudomonad species, only *P. solanacearum* is non-fluorescent (24). The fluorescent group of pseudomonads poses a particular challenge to bacterial taxonomists.

This work reviews some of the taxonomic problems of the fluorescent pseudomonads. One purpose of this research was to provide basic information on secondary metabolite production by fluorescent pseudomonads obtained from a variety of host plants. In particular, a search for the number and activity of antibacterial, antimycotic and phytotoxic agents and information to clarify relationships or synonomy within these classes of compounds produced by these bacteria was conducted. Numerical techniques were employed to provide a framework of relationships between the strains examined and not necessarily to create a new taxonomic classification scheme or revise the placement of taxa within the present system. A detailed description of the methods of numerical anaylsis is not presented. The reader interested in these topics is referred to the text by Sneath and Sokal (131) for an in-depth explanation of calculations and the theory of numerical taxonomy.

A second aim of this research was to identify antimycotic producing isolates of pseudomonads for possible inclusion in an applied research program using these bacteria in the treatment of Dutch elm disease (98,142). The realization that certain strains of fluorescent pseudomonads synthesize antimycotics has made possible a

novel approach by this laboratory for the treatment of Dutch elm disease. The causal agent of the disease, Ceratocystis ulmi, is inhibited by certain strains of the fluorescent pseudomonads. During the investigation of the possible relationships between the fluorescent pigment and antimycotic production, it was discovered that Fe(III) dramatically alters the expression of microbial antagonism by these plant-associated pseudomonads.

The isolation and characterization of an antibiotic factor from the fluorescent pseudomonad strain M27m was undertaken. This strain had been selected for use in the Dutch elm disease control program (140). The results of these experiments also indicate the importance of iron in the antibiotic production of this strain. A comparison is made of this antibiotic factor with the pseudomonad phytotoxin, syringomycin (SR), which possesses wide spectrum antibiotic activity (22). This partially chemically characterized phytotoxin and antibiotic is produced by P. syringae van Hall 1902 (46). The identity of the previously unknown basic amino acid residues of SR, a hexapeptide, was established to aid in the comparison.

Included in the pseudomonad isolates tested and characterized taxonomically in this study were two known

strains which promote plant growth (69). Recently, Schroth and Hancock have reviewed plant growth stimulation by "rhizobacteria"(125). Such bacteria aggressively colonize the roots of plants and may be considered beneficial, deleterious or neutral to the plant (125). Most of these bacteria belong within the fluorescent pseudomonads, with characteristics similar to P. putida and P. fluorescens.

Plant pathologists refer to soils where soil-borne pathogens are absent or occur at a low frequency as disease suppressive soils. Cook and Rovira (18) in a study of soil bacteria in the biological control of Gaeumannomyces graminis implicated pseudomonads as possible agents in the mechanism of suppressive soils. Recently, Kloepper et al. (68) proposed a mechanism of suppressiveness as the accumulation of iron transport chelators (siderophores) secreted by fluorescent pseudomonads which are antagonistic toward rhizosphere pathogens. Scher and Baker (124) reported that small amounts of Fusarium-wilt suppressive soils transferred to sterile soils conferred suppressiveness. A Pseudomonas sp. isolated from the suppressive soil was found to lower the incidence of disease in a disease "conducive" soil.

These examples of applied research: treatment of

Dutch elm disease, plant-growth promoting rhizobacteria and disease suppressive soil studies, involving the fluorescent pseudomonads, emphasize the importance of these potentially useful bacteria. An understanding of the mechanisms of microbial antagonism and microbial-plant interactions, particularly in respect to the role of iron, is shown to be a vital aspect for consideration in such applied research programs.

After a description of the materials and methods, the major aspects of this dissertation are presented. These aspects are the presentation of the research on the systematic analysis of the isolates, the concomitant studies of microbial antagonism and iron interaction and the initial studies of antibiotic isolation and characterization. Following these chapters, a summary and prospectus on pseudomonad-plant-iron relationships proffer hypotheses formulated from a knowledge of the data presented.

MATERIALS AND METHODS

Source of Organisms

All bacteriocin indicator strains used in the study were received through the generosity of Dr. A. K. Vidaver, Department of Plant Pathology, University of Nebraska, Lincoln, Ne, 68583. The indicator strain designations and source of original isolation are shown in Table 1 with the exception of strain Ps 17. Strain Ps 17 was used as an indicator in only selected experiments of this study because of problems relating to its growth. Strain Ps 17 was originally isolated from corn by Dr. Vidaver and identified as a *P. syringae* strain. The test strain no. 119 was received from Dr. Vidaver designated as *P. syringae* HS 191. The fungus, *Geotrichum candidum* Link ex Persoon, used as an antibiotic indicator for antibiotic activity or the pseudomonads and test strain no. 121, originally designated *P. syringae* 268, were the gifts of Dr. D. C. Gross, Department of Plant Pathology, Washington State University, Pullman, WA, 99164. A second antibiotic indicator organism, *Ceratocystis ulmi* (Buisman) C. Moreau designated Cu5F, was obtained from Dr. N. VanAlfen, Dept. of Biology, Utah State University, Logan, UT, 84322. The red yeast, a *Rhodotorula* sp., was obtained from a culture maintained within the Department of Plant Pathology,

Table 1. Geographic, host plant and donor sources of bacteriocin indicator strains.

ORIGINAL SOURCES OF INDICATOR STRAINS USED IN BACTERIOCIN ASSAY[a]

STRAIN IDENTIFICATION	STRAIN DESIGNATION	SOURCE		HOST PLANT ORIGIN
		DONOR	GEOGRAPHIC	
P. SYRINGAE	PS-Co1[b]	D.P. COYNE	COLOMBIA	SOYBEAN
P. SYRINGAE	PS14	J.H. HAAS	CANADA	BEAN
P. SYRINGAE	GN2	A.K. VIDAVER	NEBRASKA	BEAN
P. PHASEOLICOLA	HB6	M.L. SCHUSTER	NEBRASKA	BEAN
P. SYRINGAE	Ps281	NCPPB	ENGLAND	LILAC
P. SYRINGAE	PSC-1B	A.K. VIDAVER	NEBRASKA	CORN
P. GLYCINEA	PG-1T	A.K. VIDAVER	NEBRASKA	SOYBEAN
P. SYRINGAE	PS6	A.K. VIDAVER	NEBRASKA	SOYBEAN

[a]ADAPTED FROM VIDAVER ET AL: BACTERIOCINS OF PHYTOPATHOGENS *CAN. J. MICROBIOL. 18:* 705-713, 1972.
[b]CYTOCHROME OXIDASE POSITIVE; PATHOGENIC

Table 2. Source of test isolates.

SOURCE AND NUMBER OF PSEUDOMONAD ISOLATES	
SOURCE OR HOST PLANT	NO. OF ISOLATES
BARLEY	42
SAFFLOWER	35
SORGHUM	20
WHEAT	18
SAINFOIN	17
BEAN	17
SOIL	9
TOMATO	2
ELM	2
MISCELLANEOUS	9
UNIDENTIFIED	5
TOTAL	176

Montana State University, Bozeman, MT, 59717.

Most test isolates of fluorescent pseudomonads were freshly isolated from infected seed and plant tissue of a variety of plants grown in Montana (Table 2). The majority of these isolates were obtained from Mr. Darrel L. Jacobs, Ms. Cindy Orser and Mr. Hee K. Kim, Department of Plant Pathology, Montana State University, Bozeman, MT, 59717. Detailed isolation procedures have been described by Jacobs (62). All isolates produced fluorescent colonies on King's Medium B under long wave-length (366nm) u.v. light and were stored in otherwise sterile water at 4°C and at -20°C after the addition of glycerol (30%) to a log-phase culture in nutrient broth. The pseudomonad strain, M27m from the culture collection of Dr. David C. Sands (Montana State University), has been placed on permanent unrestricted deposit with the culture collection of the Northern Utilization Research and Development Division (formerly Northern Region Research Laboratory of the U. S. Department of Agriculture) and has been assigned accession No. NRRL B-12050.

P. aeruginosa PU 21 (ilv B 112, leu, str^{-1} Rif^{r}) R1033, included as a test isolate (strain no. 117) was received from Dr. George Jacoby, Infectious Disease Unit,

Dept. of Medicine, Massachusetts General Hospital, Boston, Mass. 02114. P. putida AC1012 (RSF 1010) was supplied by Dr. Suresh S. Patil, Dept. of Plant Pathology, University of Hawaii at Monoa, Honolulu, Hawaii, 96822, and is designated as strain no. 118 in the study. Pseudomonas syringae pv. tagetis strain no. 3 and G were obtained from Dr. Richard D. Durbin, Department of Plant Pathology, University of Wisconsin-USDA, Madison, WI, 53706. Fluorescent pseudomonad strains designated in this study with strain numbers 128, 129, 135, 137, and 138 were received from Dr. Trevor Suslow, Department of Plant Pathology, University of California, Berkeley, CA, 94720, designated as A1, RV3, SH5, B4, and 7SR1, respectively. E. coli 1830 pro^- met^- (pJB4JI) was received from Dr. Jesse Jaynes, Department of Plant Pathology, Montana State University, 59717. P. fluorescens P5F, a producer of pyrrolnitrin and pyoluteorin (antibiotics), was obtained from Dr. Charles R. Howell, National Cotton Pathology Research Laboratory, P. O. Drawer JF, College Station, TX, 77841.

Media

The media used in isolation of fluorescent

pseudomonads from plant materials were BCBRVB medium (123) and King's Medium B with or without 75 mg/l cycloheximide (66). Following isolation and at least one growth period on King's Medium B, a single colony-forming unit (cfu) was transferred to a master plate in preparation for replicate plating. The medium for master and submaster plates consisted per liter of the following: Yeast extract (1.0 g), glycerol (3 ml), Na_2HPO_4 (4.2 g), KH_2PO_4 (2.7 g) and agar (12 g). The replication procedure between experiments is shown in Figure 1. A custom-made multipoint replicator was used after the manner of Lederberg and Lederberg (75). In cases where isolates presented both a translucent and opaque colony type, the opaque colony was selected for testing. The fungi and yeast were assayed for sensitivity to antibiotics produced by the fluorescent pseudomonads, on a modified mineral salts medium (27) in which 1% glucose replaced 1% glycerol as the carbon source in the medium. This medium, referred to as Dye's medium, contained 1.2% agar and where indicated, 0.1% vitamin free casamino acids (Difco Laboratories) or 15 mM histidine (free base). In cases of iron supplement, the medium contained 10 mg/l $FeCl_3$ which was added after autoclaving from a filter sterilized, fresh 0.1% aqueous solution. Dye's medium with

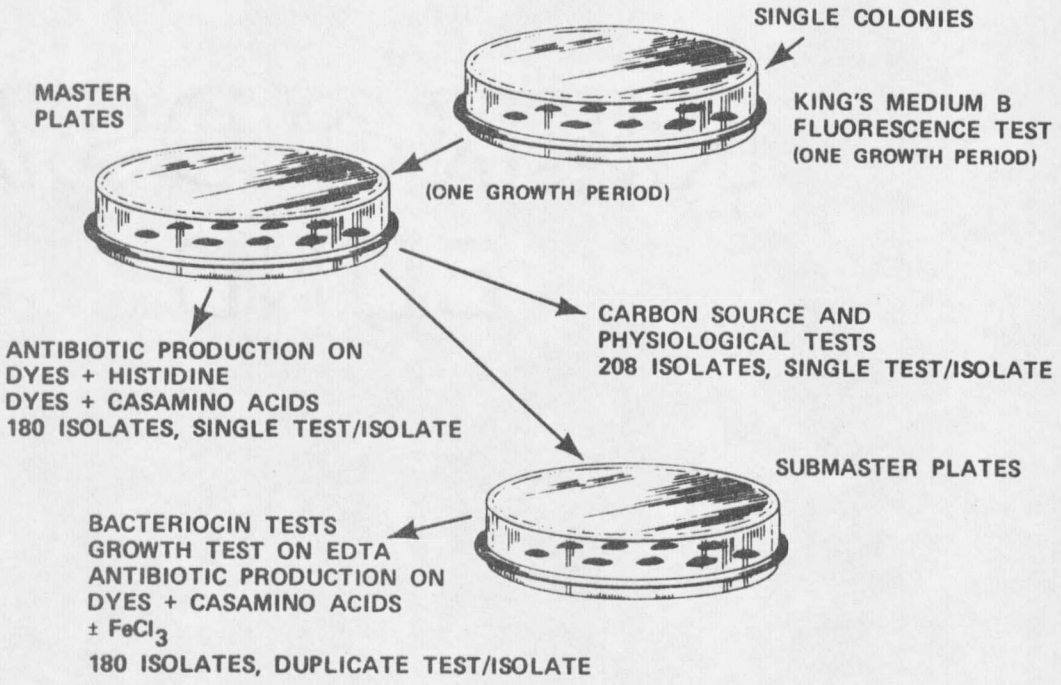

Figure 1. Replication procedure.

casamino acids containing EDTA (ethylenediaminetetraacetic acid) was prepared by addition of the disodium form to the medium prior to sterilization to make a final concentration of 10.0 mM EDTA. Nutrient broth yeast extract (NBY) medium (154) and Dye's medium were used for bacteriocin assays. Care was taken in the preparation of media used for comparison tests of the presence and absence of iron to minimize unwanted iron contamination by using glass distilled water passed through a Millipore Milli R/Q water purifier and by using Oxoid purified agar obtained from KC Biological Inc., Lenexa, KY. No further effort was made to reduce the very low level traces of iron.

Physiological and Carbon Source Tests

The media and methods for the physiological tests and carbon utilization tests have been described (62). The carbon substrate utilization tests were completed in the following manner. Substrates for the carbon source tests were filter sterilized before addition to a minimal medium, containing per liter, $(NH)_4H_2PO_4$ (1.0 g), KCl (0.2 g), $MgSO_4$ (0.2 g) and Noble agar (12 g). Organic acid and amino acid substrates (0.1% w/v) and sugars and sugar alcohols (0.2% w/v) were separately added to the basal

minimal medium as the sole carbon source with the pH adjusted to 7.2. Incubation was at 27°C with growth determined in comparison to inoculated replicate plates containing no added carbon source. All collected isolates (total 208) were replicated in these studies with at most 16 colonies/plate (Figure 1).

Bacteriocin Assay

The bacteriocin assay procedure as outlined by Vidaver, et al. (156) consisted briefly of the steps shown in Figure 2. A total of 180 bacterial test isolates with at most 10 different test colonies per plate were replicated from submaster plates (Figure 1) onto three media: NBY medium and Dye's medium containing casamino acids with and without the $FeCl_3$ addition. After incubation for 48 hours at 26°C, the plates were inverted over chloroform soaked toweling for approximately 5 minutes to kill the test isolate bacteria. The plates were aired with lids ajar for one hour in a laminar flow hood. Melted NBY medium (0.7% agar), cooled to 45°C and seeded with 0.1 ml of a log phase culture (48 hr) of the appropriate indicator strain was pipetted (4.0 ml aliquot) onto the killed test colony plates, incubated for 24 hours at 26°C

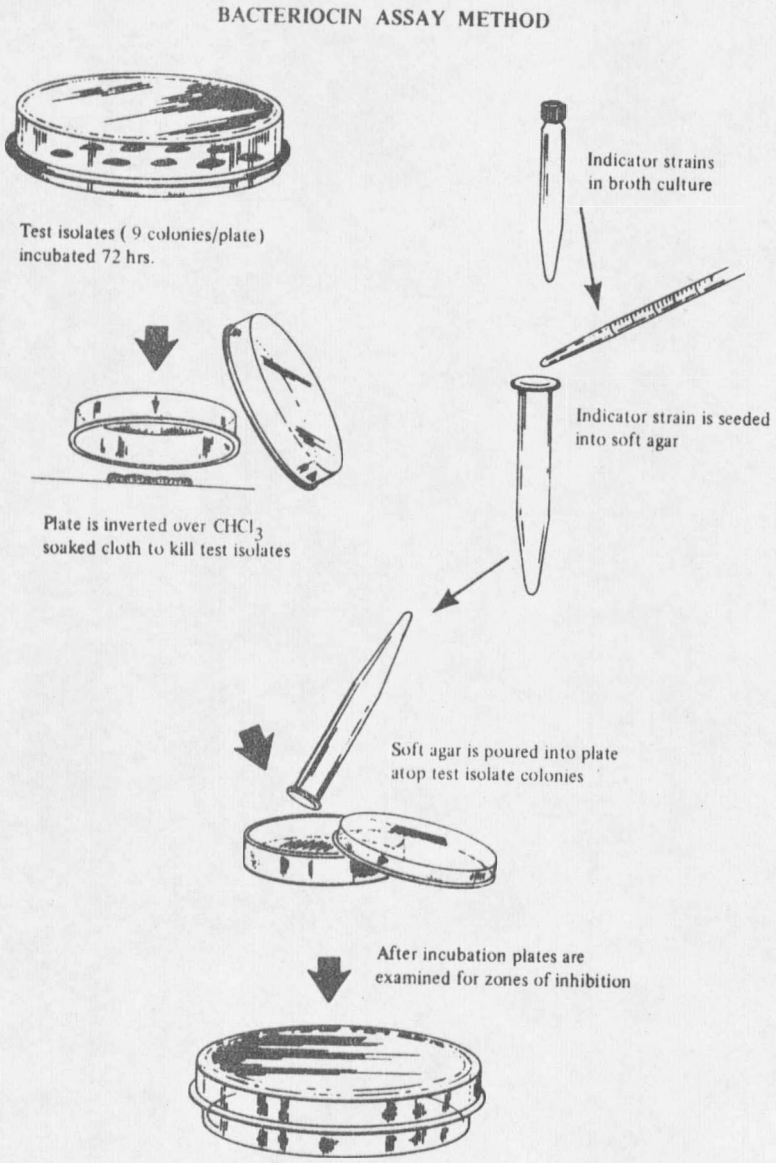

Figure 2. Pictorial representation of bacteriocin test procedure.

and observed for zones of inhibition. The type of zone, whether turbid or clear, and the distance from the colony edge to the edge of the zone was determined. Duplicate test plates of each medium and test isolate were made for each of the 8 indicator strains tested. NBY medium was used in all overlays.

Eucaryote Inhibition Screen

Ceratocystis ulmi, *Geotrichum candidum* and the *Rhodotorula* sp. were maintained at room temperature on potato dextrose agar (Difco). The arthrospores and cells of *Geotrichum* and *Rhodotorula*, respectively, were washed from one-week-old cultures with distilled sterile water and conidia were obtained from two-week-old cultures of *Ceratocystis*. The suspension of spores or cells was deposited over a pseudomonad-replicated test plate by a three-second spray with an aerosol sprayer held ca 30cm from the plate. The plates were incubated for 3 days at room temperature with observations made at one and two days of the incubation and the diameter of the zone determined on the third day. Pseudomonad test isolates for antibiotic screening were replicated from master and submaster plates. A duplicate test was performed on each isolate. Submaster

plates were necessary due to the logistics of handling the many replicates in these experiments.

Data Coding and Numerical Analysis

Results of all characterization tests (Table 3) were coded in one of two mutually exclusive states as 1 for positive and 0 for negative and infrequently 9 for no test made. The total tests included physiological and carbon utilization tests, eight bacteriocin indicator tests on each of three media, fungal and yeast antibiosis screens and miscellaneous phenotypic tests for a final matrix of 113 features.

Data were collected from 208 pseudomonad isolates for physiological, phenotypic and carbon utilization tests and examined after coding using both the simple matching coefficient, S(SM), which includes both positive and negative similarities (133) and the similarity coefficient, S(J) or Jaccard coefficient, which excludes negative matches (128). Sorted similarity matrices and dendrograms of strain similarities were obtained by unweighted average linkage clustering (131) for both sets of coefficients. The final matrices consisted of 73 characterization tests and 208 strains.

Table 3. Types of classification tests.

CLASSIFICATION TESTS—113 TOTAL

I. SOLE CARBON SOURCE—UTILIZATION TESTS

- SUGARS AND GLYCOSIDES
- SUGAR ALCOHOLS
- POLYSACCHARIDES
- AMINO ACIDS AND DERIVATIVES
- AROMATIC ACIDS
- FATTY ACIDS AND DERIVATIVES
- ORGANIC ACIDS
- MISCELLANEOUS

II. BACTERIOCIN TESTS ON 3 MEDIA USING 8 INDICATOR STRAINS

III. ANTIBIOTIC INDICATOR OVERSPRAYS ON 3 MEDIA

- *Geotrichum candidum*
- *Rhodotorula*
- *Ceratocystis ulmi*

Information was collected for antibiotic production, bacteriocin production and growth on a medium containing EDTA from 180 isolates among the original 208 isolates. As a result of observing both fungicidal and fungistatic zones of inhibition, as well as a question as to whether a bacteriocin inhibition zone was not a result of another metabolic process (ex. acid production), two formats for coding were instituted. Only a fungicidal zone was considered a positive test in set A format; whereas, both fungicidal and fungistatic zones were considered positive tests in set B format. Similarly, a bacteriocin zone of inhibition 1mm or less from the colony edge as described was coded as a negative test in set A format and a positive test in set B format. Bacteriocin zones of inhibition were also observed to be either clear or turbid in appearance, no distinction was made between these types of zones except where noted later in this discussion.

Numerical analysis was conducted for the antibiotic and bacteriocin production tests in a comparable manner as with the physiological and carbon utilization tests. The 25 bacteriocin tests were analyzed by generating dendrograms and matrices (25 tests x 180 strains) for both sets of data, A and B, and also both types of similarity

coefficients. The 25 bacteriocin tests were then combined with antibiotic production, fluorescence and EDTA growth tests (15 total) and analyzed in concert, giving matrices with 40 tests and 180 strains. These tests were also placed with the 73 physiological and carbon utilization tests which generated dendrograms and matrices for 113 tests and 176 strains with both types of formats and coefficients. Four strains were omitted in this final analysis because of incomplete data.

The 25 bacteriocin tests, which consisted of tests on 180 isolates using 9 indicators for test isolates growing on the NBY medium, 8 indicators each for isolates growing on Dyes's plus casamino acids with or without $FeCl_3$, were coded in an additional fashion to account for the clear and turbid appearance of zones. Each indicator on each medium was treated as if it were two tests. One test, which if positive (indicated by 1) represented a clear zone for that indicator on the specified medium and the second test, which represented, if positive, a turbid zone for the same indicator. The bacteriocin tests coded in this manner (50 tests) were combined with the antibiotic tests and the EDTA growth test (total 13 tests) to genereate matrices and dendrograms with set A and B data and both types of

coefficients for a total of 63 tests and 180 strains.

All computations were completed using program packages made available on the University of Maryland UNIVAC 1108 Computer, which in addition to the computations described included a feature frequency analysis on the sets of data using the simple matching coefficients for matrices formed by the 113 tests and 176 strains. All data were placed on computer cards after the appropriate coding and forwarded to Dr. Bruce Gunn in the laboratory of Dr. R. R. Colwell, Department of Microbiology, University of Maryland, College Park, MD, 20742, for computer analysis.

Transposition of Tn5

The method used for transfer of the drug resistance transposon, Tn5, to a fluorescent pseudomonad (strain no. 115 of this study, isolated from pear and originally designated DC 323+ in the bacterial collection of Montana State University, Department of Plant Pathology) was adapted from that of Beringer et al. (8). E. coli 1830 pro⁻ met⁻ (pJB4JI) was used as the donor of Tn5. The plasmid, pJB4JI, is a broad host range plasmid which conrers resistance to gentamicin, spectinomycin and low level streptomycin. Mu phage has been inserted into the plasmid, within Mu there is Tn5 which confers kanamycin and neomycin resistance.

E. coli 1830 was transferred from a single colony on LB medium containing 200 µg/ml kanamycin monosulfate to 10 ml of nutrient broth containing 200 µg/ml kanamycin and incubated at room temperature on a rotorary shaker (125 rpm) overnight (16 hrs). Pseudomonas strain no. 115 was treated in an identical manner but grown in Dye's medium containing 15 mM histidine. Preparation for the mating mixture was made by pelleting the E. coli culture, washing it twice in nutrient broth and resuspending it in 2.0 ml of this broth. The pseudomonad culture was pelleted and

resuspended in 2.0 ml sterile water. Aliquots (1.0 ml each) of the donor and recipient strains were mixed and filtered in a swinnex type syringe filter (0.22 µm Millipore filter). Aliquots (1.0 ml) of each parent were mixed with 1.0 ml sterile water and also placed on filters. Filters were transferred to an agar plate of LB medium with incubation at 27°C for 8, 24, 48, and 72 hours. The filters were removed from the plates and the cells were resuspended in 1.0 ml sterile water. Selection and counterselection in this cross of an auxotrophic donor and prototrophic recipient was completed by plating 0.1 ml aliquots onto Dye's medium containing 15 mM histidine and 200 µg/ml kanamycin. Parental strains were plated onto the selective medium and onto both Dye's medium containing only histidine and LB medium for observation of the possible spontaneous drug resistant mutants and confirmation of auxotrophy in the donor. Observed transconjugants were streaked onto the selective medium containing 100 µg/ml kanamycin and 100 µg/ml neomycin. Single colonies of each transconjugant were transfered onto Dye's medium plus histidine and oversprayed with Geotrichum after a six day growth period.

Ice Nucleation Tests

Tests for ice nucleation activity of bacterial strains within the present study were conducted following completion of the numerical analysis. The 72 strains tested were streaked from stored cultures and subcultured onto Pseudomonas F agar for shipment. These strains were sent to Ms. Cindy Orser, Department of Plant Pathology, University of California, Berkeley, CA, 94720, for tests of ice nucleation activity at -5° and -9°C (99) in the laboratory of Dr. Steven E. Lindow.

Phytopathogenicity Tests

A test of 125 isolates of fluorescent pseudomonads contained in the systematic analysis study was conducted for phytopathogenicity in African marigolds using a cultivar moderately susceptible to a bacterial leaf spot disease caused by Pseudomonas syringae pv. tagetis (142). Seeds of Tagetes erecta L. variety 'Crackerjack' were obtained locally. Seeds were germinated and the seedlings transplanted to six inch azaela pots (2 plants/pot) containing a sterilized soil and sand (1:1 v/v) mixture. Plants were grown in the green house under no auxilliary lighting. The isolates to be tested were inoculated upon

separate occasions. March 31, 1981, and June 26, 1981, in two groups of 53 and 72 different isolates, respectively, along with two control strains of Pseudomonas syringae pv. tagetis (No. 3 and G). A strain was prepared for inoculation by resuspending in sterile water colonies previously streaked on King's Medium B. The cell density was determined using a Klett-Summerson colorimeter with No. 54 green filter. An aliquot of the suspension was used immediately for a hypersensitivity test in Nicotiana tabacum cv. 'Burley' (122) with the first set of strains. The control strains were inoculated into both marigolds and zinnia (Zinnia elegans Jacq. cv. 'Instant Glamour') at densities between 50 and 300 Klett units. One plant per pot was inoculated with a test isolate. The second plant was inoculated in an identical fashion with the diluent, sterile water. Wound inoculation of 21 day-old plants was made by puncture of a 1.0 ml tuberculin syringe needle (25 gauge) through the stem of individual plants approximately 3-5 cm below the apical meristem. A droplet of inoculum was formed on the beveled orifice of the needle prior to the stem puncture. A second droplet was formed after the needle had protruded from the other side of the stem, which upon removal of the needle allowed a portion of the second

droplet to be drawn into the plant. Observation of the plants for symptom development was made daily for three weeks following inoculation.

Antibiotic Isolation and Analysis

Culture Conditions and Extract Preparation.

Pseudomonas strain M27m was grown in shake culture (180 rpm) in 2.0 liter Erlenmeyer flasks containing 1.0 liter of either Dye's broth culture medium containing 15 mM histidine both with and without supplementation of 10 mg/ml $FeCl_3$ or Dye's plus 0.1% casamino acids with and witnout the iron addition. Incubation was for 4 to 5 days at ambient room temperature and at 18°C. Such cultures were seeded with a 10 ml overnight culture (same medium) of M27m taken from colonies off Dye's plus histidine agar plates or Pseudomonas F agar (Difco) plates maintained at the optimal growth temperature of most fluorescent pseudomonad species of 27°C.

Preparation of cell-free supernatant liquids included the addition of two volumes of cold distilled acetone to the cell culture followed by centrifugation (15,000 x g, for 10 min) to remove cells, cell debris and precipitated protein. Twenty-fold concentration of the supernatant

liquor was achieved by rotary flash evaporation at 35°C. In some cases, this was folowed by extraction of this concentrated crude supernatant liquor with 3 volumes of glass distilled n-butanol. The n-butanol fractions were collected and combined with an equal volume of deionized distilled water. This combination of solvents approximates a binary azeotropic mixture which was rapidly concentrated 30 fold by rotary flash evaporation at 35°C.

On a preparative scale, nine cultures (1 liter each) were treated with acetone as described, combined and centrifuged to remove cells, etc., using a Sharples model centrifuge. Acetone was removed from the supernatant liquids by flash evaporation at 35°C. A volume of approximately 8 liters of supernatant liquid was subjected to ultrafiltration using a PM-30 membrane filter in a model TCF10 high performance thin channel system (Amicon Corporation). The material retained by the filter (100 ml) was further concentrated to a final volume of 45 ml using a PM-30 membrane in a model no. 52 stirred ultrafiltration cell (Amicon Corporation). The 45 ml of retained material was then "diafiltered" with 20 volumes of deionized distilled water. The filtrate from these steps was frozen and lyophilized in a Virtis industrial model 50-SRC freeze-

dryer. The final volume represented an approximate 200 fold concentration over the original culture volume. This material was directly bioassayed for activity against Geotrichum. The dry-matter from the filtrate was bioassayed after resuspending in water.

Bioassay Method. Collected supernatant fractions, chromatography fractions, retained materials and filtrates from ultrafiltration and aqueous extracts were assayed for inhibition of Geotrichum candidum by spotting 10 µl aliquots on an agar plate of Dye's medium containing histidine followed by drying and respotting till at most a total of 50 µl had been applied as a single spot. The plate was then sprayed with the arthrospores of the fungal culture as described for screening bacterial isolates in the systematic analysis study. Observations could be made as early as 12 hours, but 24 to 48 hour observations generally helped distinguish between possible fungistatic and fungicidal activities. A report of increased detection of antibiotic sensitivity by others (52) when sensitivity tests were completed on plates made with agarose rather than agar of the same porosity was confirmed for this assay. This prompted a change to 0.8% agarose (ultra-pure high gelling temperature SeaKem agarose manufactured by

Marine Colloids Division, FMC Corporation) in plates used for the assay of fractions. This percentage of agarose corresponds to the porosity of 2.0% agar (52).

Chromatographic Methods. Following the preparation of cell free crude supernatant concentrates several chromatographic separation methods were employed in fractionation and isolation of inhibitory activity(s) against Geotrichum. The order of inclusion of these techniques in the isolation procedure is presented in Figure 3.

Conventional column chromatography was conducted using Bio-Gel P-2 (200-400 mesh) material swollen in water and packed to provide a bed 90cm x 1.4cm (i.d.) in size. Samples applied to the head of the column ranged from 1.5 ml to 5.0 ml in volume. Water served as the eluting solvent by gravity flow. The column was characterized using bovine serum albumin (66,000 MW), cyanocobalamin (1,357 MW) and L-tryptophan (204 MW) as standards. The effluent was collected in 4.0 ml fractions following spectrophotometric monitoring (Gilson Holochrome spectrophotometer) at a wavelength of 280 nm. Fractions were concentrated (4X) by flash evaporation prior to bioassay of 50 µl aliquots. A P-100 column was also

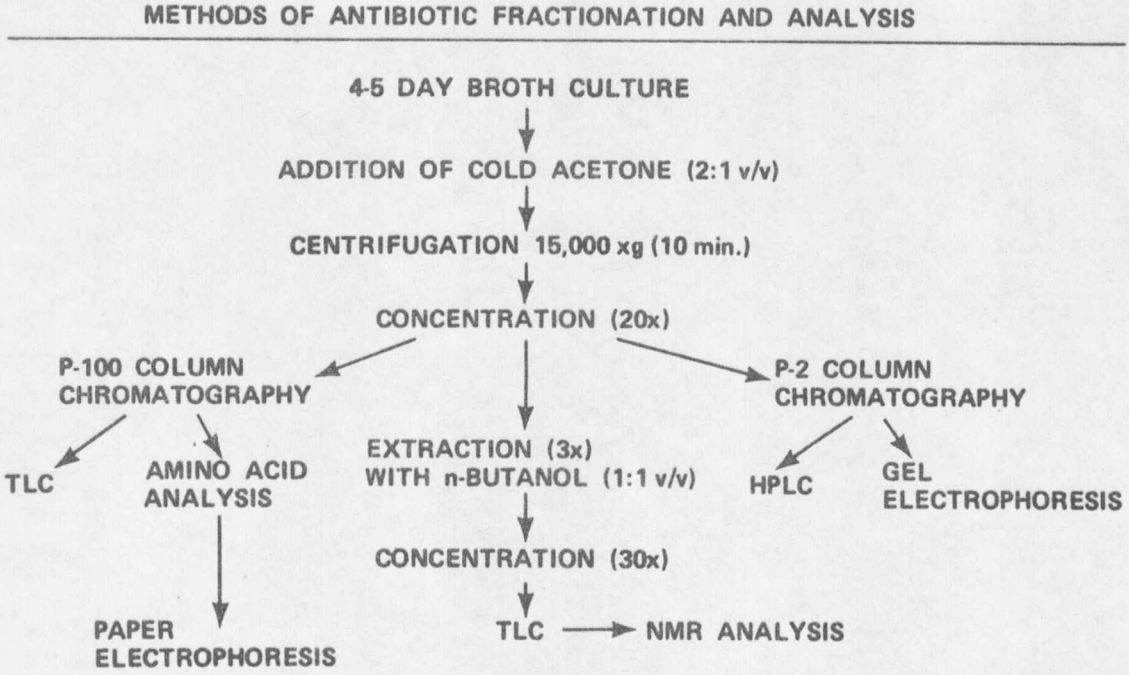

Figure 3. Schematic outline of the isolation and analysis procedures.

employed in a similar fashion but having bed dimensions of 40cm x 2.5cm (i.d.) and fractions collected in 8.0 ml volumes. Samples were generally in a volume of between 50 ml and 15.0 ml. Blue dextran (2,000,000 MW) served to locate the void volume.

High pressure liquid chromatography was utilized in both the reversed phase mode and exclusion mode. The antibiotic material from Pseudomonas strain M27m was bound strongly to microbondapak C18 packing material. Using C18 sep pak cartridges (Waters Assoc., Inc.) several solvents were used to elute biologically active material, with no success. The use of two prototype I-60 columns (on consignment from Waters Assoc., Inc.) allowed size separation of crude fractions on an analytical scale. HPLC analysis of biologically active P-2 column fractions was conducted using these columns in series. Injection and sample volume was 20 µl of the concentrated fraction (1.0 ml). Further chromatographic conditions are presented with the discussion of the resulting separation (Figure 33). Instrumentation included the Waters Associates 6000A and M-45 pump modules, a 660 gradient programming module, a U6K universal injector and 440 absorbance detector fitted with a filter for 280 nm wavelength detection.

Thin-layer chromatography was performed using silica gel, 250 µm hard-surfaced analytical layer plates (5cm x 20cm) with 254nm fluorescent indicator (R.T. Baker Co.). Development was completed in 2.5 hours in a preequilibrated chamber lined with 3MM paper and soaked in the solvent. The solvent was a mixture of n-butanol: glacial acetic: pyridine: deionized distilled water (15:3:10:12). Total sample volume applied was generally 50 µl. Several detection methods were used exclusive of observation under long and short u.v. light. These techniques included the spray reagents, 0.25% ninydrin in butanol, Folin-Ciocalteu reagent for hydroxamate siderophore detection (145) and a reagent for specific colorimetric detection of o-diphenols, and 3,4-dihydroxyphenylalanine-containing peptides (157). Detection of iron binding activity on thin layer plates was achieved by the addition of 40 µl of a 0.1% solution of $FeCl_3$ to a 100 µl aliquot of a concentrated butanol extract (Figure 3) prior to chromatography. Application of a 50 µl sample to the TLC plate, followed by development and detection using 2 N HCl and 1% aqueous potassium ferrocyanide, allowed the observation of Fe(III) binding activity as a light blue spot or band (36). An autoradiogram of the TLC plate was made in cases where Fe^{59}

in the form of $FeCl_3$ (0.2 mCi/ml) served as the iron addition to the extract as described above. This was done by exposing XR5 Kodak X-Omat RP x-ray film to the TLC plate for 2 weeks while stored at 4°C. Film development was by an automated rapid x-ray processing system.

Characterization methods. A gel electrophoretic comparison between a biologically active P-2 column fraction from a culture supernatant of strain M27m and a sample of syringomycin (SR) obtained from Dr. J. E. DeVay, Department of Plant Pathology, University of California, Davis, CA, 95616, was undertaken and completed after the manner described for SR by Gross and DeVay (46). Briefly, this consisted of the preparation of 15% native acrylamide gels (in this case 15cm x 0.5cm (i.d.) tubes were employed). The electrophoresis buffer was composed of 35 mM beta-alanine and glacial acetic acid (final pH 4.45). Migration of samples was toward the cathode in this system. Bioassay tests of diced gel sections (~0.3cm in length) were completed as described (46) but upon Dye's plus histidine agar plates.

Leucine aminopeptidase treatment of a crude extract of an M27m culture supernatant fraction involved the use of a colorimetric method to confirm enzyme activity within the

preparation. L-Leucine-p-nitroanilide served as the substrate for the enzymatic activity determination as outlined in Sigma Technical Bulletin No. 251. Other simple tests carried out on the supernatant fractions following the acetone precipitation step included immersion for 3 minutes in a boiling water bath and treatment with 12 N NaOH and retitration to neutrality. Bioassay tests of the fractions before and after treatment were executed to determine the effect of such treatments on antibiotic activity against Geotrichum.

Proton NMR analysis was performed on a 3 mg sample dissolved in 1.0 ml deuterated DMSO on a Bruker WM250FT instrument. The biologically active sample for analysis was obtained from a butanol extraction of M27m cell free culture supernatant liquor following a TLC separation (Figure 3), recovery of the active material in ethanol and concentration to dryness by flash evaporation.

Amino acid analysis of chromatographic fractions and standards was executed on a Beckman model 120C amino acid analyzer equipped with both an intermediate length column (23cm) containing type AA-15 custom research resin and a short column (5.5cm) containing type AA-27 resin. The circulating water of the column jackets was maintained at

55°C. The elution buffer consisted of 0.35 N sodium citrate with a pH of 5.25 adjusted by the addition of HCL to within 0.02 units. Caprylic acid served as the buffer preservative (0.1 ml/liter). The total flow rate of both buffer and ninhydrin was approximately 100 ml/hr. Samples (1-3 mg) were hydrolyzed in vacuo with 1.0 ml constant boiling HCl at 110°C from 3 to 15 hours. The hydrolysate was concentrated by flash evaporation to dryness after which 2.0 ml of sample buffer was added. The buffer, sodium citrate (pH 2.20, 0.2 N Na^+), of commercial preparation (Pierce Chemical Co.) served as the sample buffer. For particularly labile fractions, the concentration procedure following the hydrolysis was amended to include the following steps. To the 1.0 ml hydrolysate was added 1.0 ml deionized distilled water which was then concentrated by flash evaporation at 40°C to a volume of 1.0 ml. A second 1.0 ml volume of water was added followed by concentration to a volume of between 100 and 200 microliters. To the final concentrate was added 2.0 ml of sample buffer.

Paper electrophoresis was conducted on acid hydrolysates and single amino acid standards using an LKB Multiphor 2117 electrophoresis apparatus and Brinkmann

voltage and current regulated power supply. The technique was adapted from that of described by Emery (34). The electrophoresis buffer consisted of the mixture, pyridine: glacial acetic acid: water (14:10:930) at pH 5.0. Development was carried out on Whatman no. 1 paper at 200 volts for a 45 minute duration. Samples (25 ul) were applied from hydrolysates or standards (1 mg/ml). Visualization of spots was by means of ninhydrin or tetrazolium chloride spray (132). Hydrolysis of the deferri form of the siderophores, aerobactin and rhodotorulic acid, provided the standards, N-OH-lysine and N-OH-ornithine, respectively. These standards were used for electrophoresis and for the amino acid analysis experiments. The mono-2-nitro-1,3-indanedione salt of N-OH-lysine was also analyzed as a possible standard (121) and was provided for by Dr. Samuel J. Rogers, Chemical Station, Department of Chemistry, Montana State University, Bozeman, MT, 59717. Rhodotorulic acid was a gift of Dr. Thomas Emery, Department of Chemistry and Biochemistry, Utah State University , Logan, UT, 84322. A sample of aerobactin was kindly supplied by Dr. J. B. Neilands, Department of Biochemistry, University of California, Berkeley, CA, 94720. Authentic samples of antibiotics

isolated from pseudomonads included a 50 mg sample of sodium pseudomonate A from Dr. N. H. Rogers, Beecham Pharmaceuticals, Animal Health Research Centre, Tadworth, Surrey, KT207NT, England and a 5.0 g. sample of pyrrolnitrin from Fujisawa Pharmaceutical Co., Ltd., Osaka 541, Japan.

SYSTEMATIC ANALYSIS

Present State of the Taxonomy and Nomenclature

The unique characteristic of the bacterial strains under examination is the production of diffusable water-soluble fluorescent pigments. This phenotypic trait has served for years in the identification of certain species of the genus *Pseudomonas* (24). The fluorescent species include *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. chlororaphis*, *P. aureofaciens*, *P. cichorii* and *P. syringae* (24). The names "pyoverdine" and "pseudobactin," have been given the pigments produced by *P. fluorescens* (88,150). The pigments produced by *P. aeruginosa* have been termed "pyocyanines" (167) and the general term of "bacterial fluorescein" has also been associated with the fluorescent pigments (81). Except for only very recent studies (150), the exact structure and function of many of these compounds remains unknown. Confusion about the pigments has existed in the literature because of the inexact usage of terms, the synonomy which exists, the existence of nonfluorescent pigments, and poor identification of these fluorescent pigments (109). This situation may be a reflection of a similar state of confusion in the nomenclature and taxonomic positions of many fluorescent pseudomonads. These problems have been reviewed (24,26,28).

In brief analysis, the eighth edition of Bergey's Manual (24) restricts descriptions of species to those whose phenotype has been extensively characterized. For example, 43 nomenspecies listed in the previous edition (12), which probably included synonyms, biotypes, pathotypes, or varieties are included with *P. syringae* in its latest revision. Under this system the use of unambiguous names for organisms showing clear differences in important characters (i.e. phytopathogenicity) has been slighted (26). The basis for creating the revised edition was to overcome the difficulty in distinguishing nomenspecies based upon phenotypic tests.

The phenotypic tests used to establish and identify species have been predominantly nutritional in character and analyzed by numerical methods (78,122,134). Sands, et al. (122) pointed out the frequent error of introducing bias in classification by overweighting certain characters by a heavily imbalanced series of tests. In general, tests of carbohydrate metabolism and of a morphological nature had dominated many studies (122). Such an emphasis may have depreciated the possible significant contributions toward a distinction between species by other metabolic pathways of specific metabolites and membrane and cell wall

characteristics and enzymes. A more diversified array of tests, but still predominately dissimilatory tests, has been used in more recent systematic studies within the genus (42,67). Incorporation of such test data should aid in the betterment of the present determinative scheme (24) which characterizes only 10% of the Pseudomonas spp. described in the literature (67).

A recent study included a set of 120 morphological and physio-biochemical characters for 290 bacterial strains of the genus, Pseudomonas (67). The results of the numerical classification confirmed the view that "there exist in nature compact groups of microorganisms corresponding to traditional species, and linked by few forms (129)." Three of the phenons were composed of strains not yet identified but of possible independent species status (67). A different picture was presented by P. fluorescens. Classification of this group (phenon) was complicated by the profusion of associated and intermediate forms which made up one third of all the strains in the phenon (67). Under such conditions, these researchers opted to recognize the existence of a single species of P. fluorescens with subgroups considered as varieties or biotypes. The same recommendation has been made and adopted for P. syringae

(24,122). It appears that with the present tests the distinctions between biotypes or varieties of a single species and separate (but similar) species is most difficult to resolve within the fluorescent pseudomonads.

The phenotypic approach has been augmented in the Pseudomonas genus by "genotypic" tests, nucleic acid hybridization studies (17,109-111,116) and immunological relationships of proteins (17,135). The approaches of comparative enzymology (13) and phage sensitivity (9) have also demonstrated some utility for the pseudomonads. Although distinctions are often difficult in this group of organisms, thereby justifying the assemblage of the majority of phytopathogenic fluorescent pseudomonads under the name of P. syringae, it should be recognized that clustering of strains around certain nomenspecies on the basis of nutritional or physiological characteristics (67,78,122) or by DNA-DNA hybridization experiments (116) does occur. In contrast, many species showed no detectable DNA-DNA homology and in ribosomal RNA-DNA hybridization experiments some of the delineated sub-generic groups were found to be more similar to Escherichia coli than to each other (17).

Among the plant-associated fluorescent pseudomonads

are found both saprophytic and pathogenic strains. Nutritional and biochemical comparison of these two groups has been studied (92,108) in an attempt to develop methods to quickly delineate a pathogen from the associated microflora. In general, the pathogenic strains utilize fewer sole carbon sources (92,122), but there is relatively little information available on the relationships among the saprophytic and potential phytopathogenic members of the fluorescent group. The host of origin and type of lesion produced have a high taxonomic value to plant pathologists, but host range studies are by nature generally limited, investigators "rationally, attempt identification by other means (109)." The fluorescent pathogenic strains are frequently characterized by the absence of the arginine dihydrolase system. The loss of this system is viewed as a separate evolutionary branch of the P. fluorescens complex as defined by DNA homology studies (110). With the exception of P. cichorii, members of this branch are oxidase negative, a unique character apart from other members of the complex and also unique among most members of the genus (24). Lelliott et al. (78) considered five tests as best in differentiating plant-pathogenic nomenspecies from each other and from saprophytes. These

tests, production of levan, oxidase reaction, soft rotting of potato, presence of arginine dihydrolase, and hypersensitivity reaction on tobacco (="LOPAT"), placed all plant pathogens tested into four groups.

The interested taxonomist is referred to a monograph written by Palleroni (108) and a review of the same (101), which represents the most thorough and updated work devoted to taxonomic problems of Pseudomonas. According to this work, the possibilities of utilizing data on cell-membrane external structure in Pseudomonas remains to be exploited. Since the cell-membrane plays a major role in viability, in reactions to phage and bacteriocins, in antigenic properties and in resistance to antibiotics, such data might provide a discriminating look at the plant-associated fluorescent pseudomonads. The prospects of a selective determination scheme are heightened by the presence of external cell membrane components which vary considerably both at the species and strain levels within the genus (108).

Systematic analysis methods have been applied successfully to bacterial isolates from large mixed populations found in aquatic habitats (42), in plant litter and soil (5), in the phylloplane (51) and rhizosphere of

particular plants (45). Academic interest occasionally prompts the precise classification of bacterial isolates; however, the results are often of important practical significance. Numerical analysis of isolates allows an objective choice of strains for comparative taxonomic, ecological, biochemical or pathological studies. Numerical methods have been used widely for classification but less frequently for identification. The general problems of probabilistic identification of bacteria by computer have been elaborated upon in a series of papers by Lapage et al. (7,73,164). A program has been prepared for identification of an unknown with two-state data against a previously erected identification matrix of percent positive characters (130).

Strain Variation

Extrapolation from laboratory data to populations of wild bacteria associated with plants in nature and in states of plant disease is difficult and often results in failure. The isolation of plant-associated bacteria by plating and subculture as a pure culture in vitro removes the isolate from the challenges of the environment. Strain variation as a result of isolation may significantly affect

the plant taxonomy of associated bacteria. Costerton, Irvin and Cheng (20) have reviewed the importance of the glycocalyx in nature and disease states. They defined the bacterial glycocalyx as "those polysaccharide-containing structures of bacterial origin lying outside the integral elements of the outer membrane" in the case of Gram-negative cells (20). These structures have been largely overlooked because they hadn't been visualized by conventional light or electron microscopy and are often lost in culture; the bacterial cell surface often alters radically and quickly (23). They may serve an important role in adhesion to sister cells and to inert and tissue substrata (i.e. the phylloplane or rhizoplane of plants). Some protection is afforded by the glycocalyx from bacteriocins, bacteriophage, antibiotics and surfactants. Variable loss of the glycocalx in vitro or production under different culture conditions could affect bacteriocin and phage typing for taxonomic purposes.

The composition of the glycocalyx may include fibrous polysaccharides, globular glycoproteins and of pilin subunits of pili sufficiently long to reach the outer surface of the bacterial cell (20). A characteristic of some plant-associated bacteria (ex. rhizobia) is

specificity in colonization of the plant surface (57). The adhesion of many Gram-negative bacteria to host cells is mediated by fimibriae or pili (107).

Occasionally cultures of fluorescent pseudomonads have been unstable with respect to colony type (156). The exact nature of this phenomenon is unknown. Similarly, the mechanism underlying the instability of Pseudomonas aeruginosa segregation of mucoid and non-mucoid revertants is unknown (44). Dissociation into colony types could be a result of genetic mechanisms known to occur among pseudomonads. One mechanism could be the loss of plasmids. Plasmids in Pseudomonas have been reviewed by Chakrabarty (16). The phenomenon of a negative (or different) sector for a given characteristic arising out of a colony positive for the trait is reminiscent of biphasic variation of flagellar antigens in Salmonella (76). The flagella of most Salmonella serotypes occur in two alternative, clonally metastable phases (170). This mechanism involves a site-specific inversion event which regulates gene expression (168,169). The inversion frequency is greater than random mutation rates. This phenomenon has been also reported for the flagellar antigens of Pseudomonas aeruginosa (117). The type strain of P. aeruginosa is

found associated within the P. fluorescens complex as defined by DNA homology studies (110) which makes the speculation of such a phenomenon within the fluorescent pseudomonads appear more plausible. Phase variation of fimbriae in E. coli similar to flagellar variation has been shown to be under transcriptional control (31).

The genetics of Pseudomonas has been reviewed by Holloway et al (53,54). The genetics of the fluorescent phytopathogenic pseudomonads has been generally left unexplored. Transduction, transformation and plasmid transfer by conjugation have been demonstrated (71 and rererences therein); however, transfer of chromosomal genes by conjugation has not been shown. Conjugal gene transfer is well known in P. aeruginosa (24). In crosses between P. syringae pv. glycinea and P. aeruginosa, chromosome mobilization by plasmids allowed stable incorporation of P. glycinea genes into the P. aeruginosa chromosome (74).

A third mechanism underlying colony segregation may be implicated by the variety of characteristics for which such sectoring occurs (Results and Discussion). Formation of merodiploids occurs in 0.5 to 5% of Rhizobium lupini transconjugants (49). Differentiation of diploid transconjugants from haploid recombinants is possible where

the parents exhibit different phenotypic traits. A wide variety of segregants in different crosses could result via this process. Conjugational ability is not a prerequisite for merodiploid formation, since diploidization as a result of transformation and transduction (shown in other genera), as well as conjugation is known and seems to be independent of the mechanisms of gene transfer (49). Heumann and Springer (49) have explained well some of the important distinctions in this process: (underlining and parenthetical explanations are added)

> In all cases when the cytology of the diploid clones has been investigated, the stabilization of the exogenate (DNA separate from the genomic DNA) into the residential chromosome has been found to occur by insertion. In this respect the fate of the extrachromosomal DNA in a recipient cell is basically different whether it is homologous donor chromosomal DNA or the DNA of a plasmid. The DNA of the extrachromosomal element can be stabilized in both ways as CCC DNA (covalently closed circular DNA) forming a separate replicon or by reversible insertion into the residential chromosome, whereas during diploidization the exogenate is stably inserted into the chromosome. Plasmid DNA can be recovered by molecular preparation procedures, whereas the diploidization becomes apparent by the product of diploid genes and by the segregation pattern of endo-chromosomal recombination.

Genes stably inserted into the host chromosome may also be single copies per cell as well as genes creating the

diploid state. Diploidization may not only underlie the phenomenon of sectoring colonies but may act as a mechanism in gene transmission in the formation of intermediate strains. The taxonomic problem of the plant-associated fluorescent pseudomonads might be an indication of the results of such a mechanism.

Variation or instability among the fluorescent pseudomonads, whether it arises as a result of plasmids, phase variation of pili and flagella, merodiploidy, or a high mutation frequency is of concern to taxonomists and plant pathologists alike. Further study of this phenomenon and the importance of the glycocalyx and its role in nature is definitely warranted.

Results and Discussion

Comments on the Data and Methods. The systematic analysis of isolates reported here was undertaken to provide a framework for the examination of microbial antagonism and influences which host origin may have on phenotypic test results for plant-associated fluorescent pseudomonads. Numerical methods allowed the integration of data gathered from a wide variety of tests as noted in Chapter 2. A list of all the individual tests (113 total)

and the percent positive responses (calculated as part of the feature frequency analysis for set B data) for the 176 strains tested is given in the appendix (Table 20). Source of origin and strain numbers for all isolates included in this study may also be found in the appendix (Table 21).

Dendrograms and matrices were completed using unedited data. Data editing usually involves removing from the feature by strain data matrix, features that lack discriminating power; i.e., those tests that are either all positive or all negative for every strain in the study (158). This results in more discrimination of groups witnout altering the order of strains. Elimination of such features avoids scale compression. The application of the results of this systematic analysis in providing a framework can be attained with unedited data, without the incurrence of further significant expense or loss in the ability to select different strains for further study of microbial antagonism or plant pathogenicity. All strains were positive for 6 features and negative for 12 features. The unedited feature by strain matrix of this study results in a 15.9% minimum similarity among all strains (for the simple matching coefficient) since 18 features are nondiscriminatory [(18/113) x 100 = 15.9%]. Negative

matches are ignored with the Jaccard coefficient, therefore a minimum similarity among all strains of the unedited matrix of 5.3% is calculated (158).

The editing of missing data is important to eliminate strains which will move individually from the established order determined by one type of coefficient when a different coefficient is used. In practice, others have found that missing strain data between 10% and 25% is satisfactory for most studies (158). No strain in the final strain by feature matrix (176 x 113) lacks data for more than 26 tests (23%). For the 57 strains which lack data for at least one feature, a mean of 6.0 missing features (5.3%) is calculated. In the second case, a large amount of missing data for a given feature is not serious provided not too many features lack data (158). Of the 113 features employed in the final matrix, 62 lack data for at least one strain. The mean of these features lacking strain data is 5.7 (3% of 176 strains) and none lack more data than for 14.2% of the strains (from data found in the appendix, Table 20).

Analysis of Carbon Utilization Tests. An examination of the feature frequency analysis data (appendix, Table 20) revealed that all strains could use L-alanine, fructose or

citrate as a sole source of carbon. Levulinate, malonate, palmitate, stearate, L-tartarate, starch, trigonelline or hydroxybenzene (0.1% w/v) did not support growth of any strain. Many isolates were not discriminated from one another by the 73 physiological and carbon source tests. The dendrogram constructed by unweighted linkage of the similarities, S(J), determined for 208 isolates (Figure 4) only distinguished a total of 90 unique strains (43%).

The ordered similarity matrix presented in Figure 5 reveals a major division in the strains. Numbered groups in this figure are for convenience in referring to the matrix and do not necessarily represent clusters. Division I encompasses groups 1-3. Groups 1 and 2 comprise a cluster with approximately 80% or greater interstrain similarity. Within group 1 is strain no. 121 identified as P. syringae strain 268 and strain no. 116 also designated Pseudomonas strain M27m which is used as an experimental control agent for Dutch elm disease. Division I strains are fastidious in their use of carbon compounds; however, group 3 strains appear as intermediates with some characteristics of Division II strains which are nutritionally versatile in character. Within group 3 is P. syringae HS191 (strain no. 119). Group 4 is composed

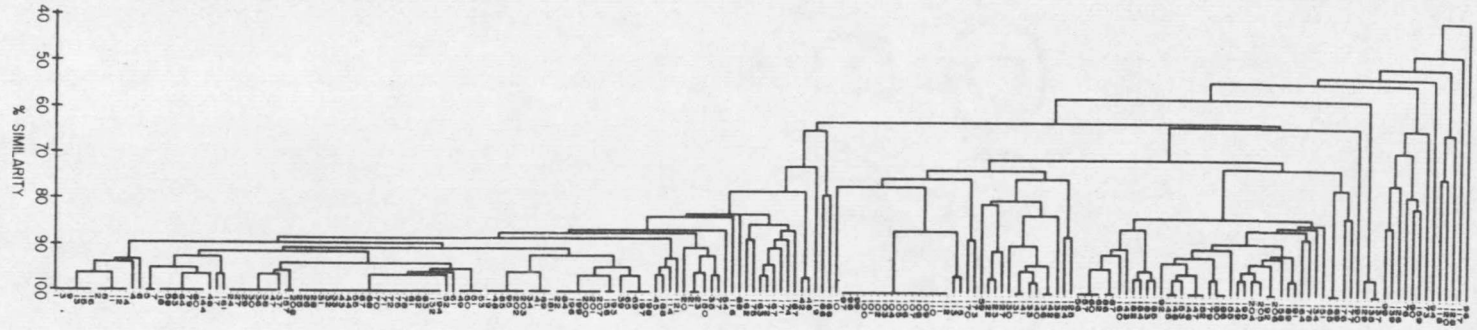

Figure 4. Dendrograms constructed by average linkage of similarities, S(J), for 208 isolates with 73 physiological and carbon source tests.

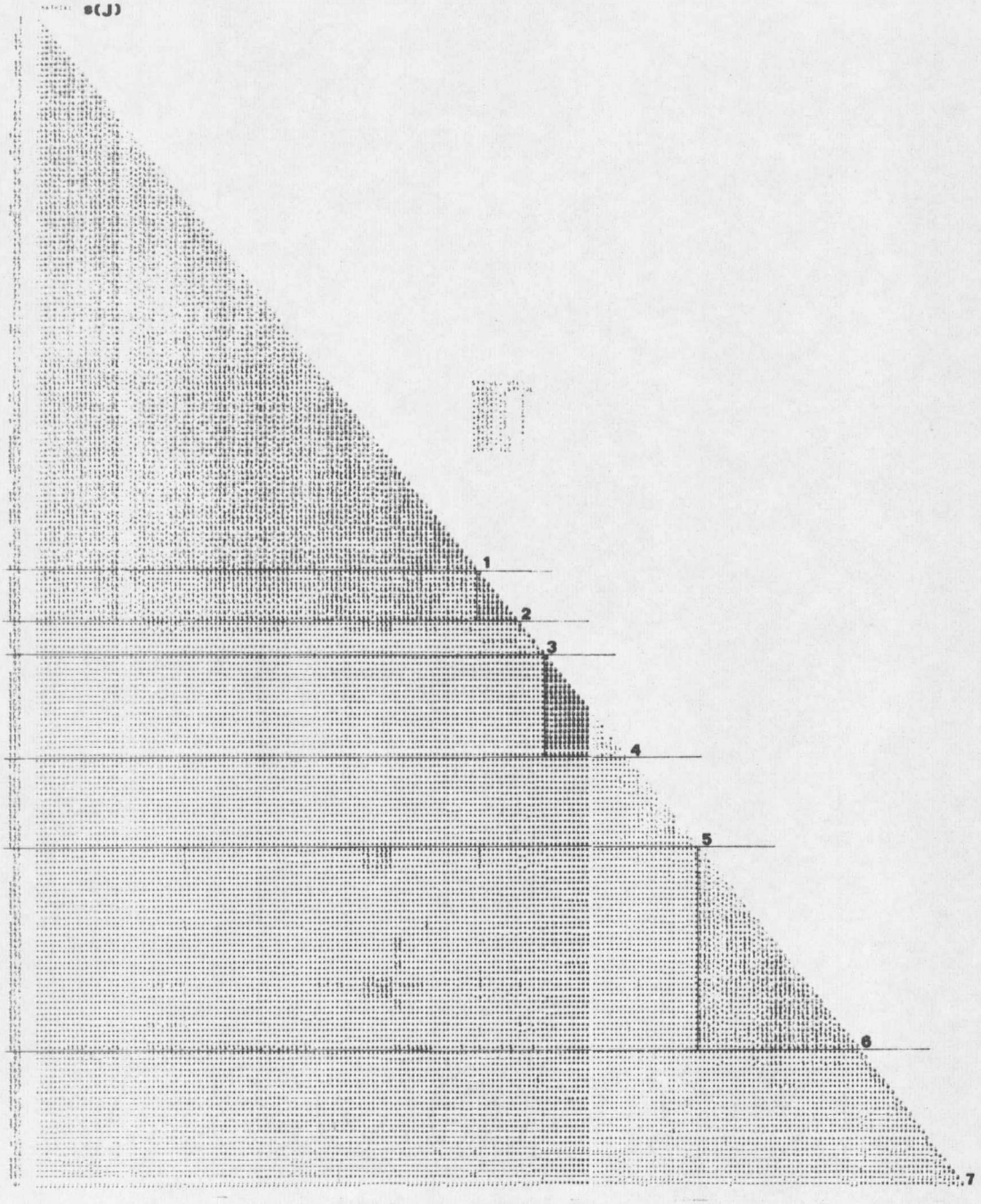

Figure 5. Matrix of similarities, S(J), for 208 isolates based on 73 physiological and carbon source tests.

entirely of strain no. 115 (originally DC 323+) and its putative transconjugants from a cross with E. coli 1830. These strains are discussed later in this chapter. Group 5 contains Pseudomonas 7SR1 (strain no. 138), an isolate considered deleterious to plants (125). In contrast, plant growth promoting pseudomonads, A1 and B4 are found in group 7 with strain numbers 128 and 137, respectively. Pseudomonas strains RV3 (strain no. 129) and SH5 (strain no. 135) obtained from the same collection (Dept. of Plant Pathology, Univ. of California, Berkeley) are also contained in group 7. These tests made no distinction between plant growth promoting pseudomonad strain B4 and Pseudomonas SH5. The plant growth promoting pseudomonads are considered to be in the Pseudomonas fluorescens group with some characteristics of P. putida (124). P. aeruginosa Pu 21 and P. putida AC1012 (strain nos. 117 and 118, respectively) are also found in group 7, which is a grouping of heterogeneous strains. The ordered similarity matrix calculated using simple matching coefficients, S(SM), for this study is found in the appendix (Figure 37).

The order of reference strains and the nature of carbon utilization among the isolates clearly indicates Division I strains would be classified within the

Pseudomonas syringae group with most of Division II strains classified as P. fluorescens. All of Division II strains would likely be in the P. fluorescens complex as defined by DNA homology studies (110).

Analysis of Combined Tests. As described (Data Coding and Analysis, Chapter 2), the total tests which included physiological and carbon source tests, eight bacteriocin indicator tests on each of three media, fungal and yeast antibiosis screens and miscellaneous phenotypic tests were combined and analyzed as a 113 feature by 176 strain matrix for both S(SM) and S(J) similarities. This was done for data coded in two formats. Set A format coded only fungicidal zones and bacteriocin zones larger than 1mm from the edge of the colony. Set B included recognition of both fungicidal and fungistatic activities in zone production and all bacteriocin zones regardless of size. The final matrix for set B will be described in detail and used as a basis to make comparisons with other test combinations.

The dendrogram constructed from set B similarities, S(SM), for 176 strains and 113 features (Figure 6) shows the pseudomonad isolates grouped into distinct clusters, albeit with a high degree of similarity. The two most divergent strains were nearly 70% similar by simple

matching over all features of the unedited data. Five intermediate strains are omitted from the dendrogram in Figure 6 for clarity in the presentation of clusters. These strains are however listed in the similarity matrix (Figure 7) and the dendrograms listing all ordered strain numbers (Figure 8).

The type of host plant from which isolates originated may be related to the clustering observed (Figure 6). Cluster E contains strains predominately isolated from wheat and barley (25/33, 76%); whereas, cluster A and B are formed of isolates from sorghum, safflower and bean (59/92, 64%). The relationship is not strict, but a trend is evident. It has been suggested that the host plant may serve as a selective medium for plant-associated pseudomonads (50). Isolates with demonstrated host specificity would be expected to reflect this attribute in their nutritional spectrum, if the appropriate substrates could be determined. Host specificity for these isolates is not known. A comparison of the position of these clustered strains within the groups defined for the physiological and carbon source tests (Table 4) identifies clusters A through D as members of division I, the nutritionally fastidious strains. Division I contains the

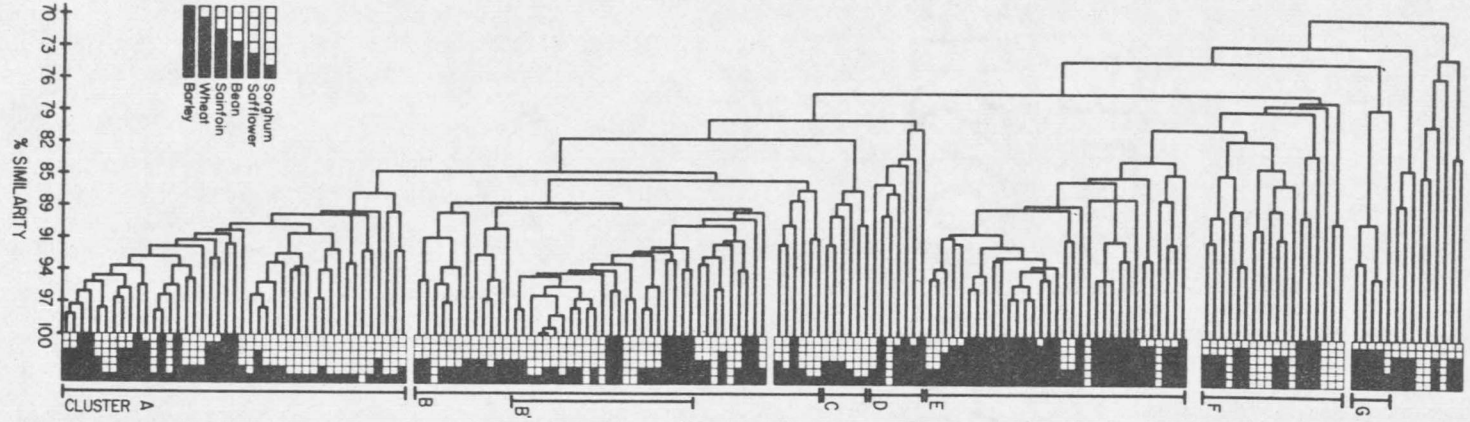

Figure 6. Dendrogram indicating host source constructed by average linkage similarities, S(SM), for 176 isolates with 113 features.