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Arthur Robert Anderson for the degree of Doctor of Philosophy

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Title: TAXONOMY AND HOST SPECIFICITY OF THE GENUS AGROBACTERIUM

Abstract approved: Redacted for privacy

Larry W. Moore

Redacted for privacy

Raymond Seidler

One hundred seventy-eight *Agrobacterium* isolates, 120 United States (U.S.), 26 Australian and 32 of miscellaneous origin, were characterized for their reactions to 20 biochemical and physiological tests. Isolate pathogenicity and degree of host specificity, utilization of nopaline and octopine, and sensitivity to agrocin-84 also were determined. The U.S. isolates were quite similar, but not identical in character to the 26 Australian isolates. Nearly all the isolates were separated by 15 diagnostic tests into two homogeneous groups, designated 3-ketolactose positive [K(+)] and 3-ketolactose negative [K(-)]. Sixteen isolates did not conform to either group description. Isolates of the species *A. tumefaciens* and *A. rubi* were biochemically, physiologically and pathologically indistinguishable. Five of eight confirmed *A. rhizogenes* isolates induced tumors in addition to hairy root and were all K(-) in character. A taxonomic proposal

for the genus *Agrobacterium* is presented wherein two species would be recognized.

All agrobacteria were inoculated to 11 known crown-gall hosts to determine their host range. The host specificity of all isolates varied greatly with no isolate infecting all host plants. Sixty-six percent of the pathogenic isolates infected six to eight of 11 hosts tested while 3% infected only the host plant from which they were originally isolated (homologous host). However, the host range of an isolate was not influenced by the plant of isolation. Evidence for change in an isolate's host specificity pattern while in culture is presented and discussed relative to its relationship with the Ti plasmid. Tomato and datura were infected by more of the pathogenic agrobacteria (81% each) than any other of the 11 host species tested, thus serving as the best indicators of an isolate's pathogenicity.

Low correlations were observed between all combinations of five isolate characters; pathogenicity, 3-ketolactose reaction, nopaline and/or octopine utilization and agrocin-84 sensitivity. Of 32 possible permutation groups of the five characters examined, isolates belonging to 25 groups were identified. Isolates were randomly distributed among all 25 groups regardless of geographic origin or plant species of isolation. Possible reasons for the lack of high correlations between the plasmid-coded characters are discussed.

# Taxonomy and Host Specificity of the Genus *Agrobacterium*

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Redacted for privacy

Associate Professor of Botany and Plant Pathology
in charge of Plant Pathology major

Redacted for privacy

Associate Professor of Microbiology
in charge of Soil Microbiology major

Redacted for privacy

Chairman of Department of Botany and Plant Pathology

Redacted for privacy

Chairman of Department of Microbiology

Redacted for privacy

Dean of Graduate School

Date thesis is presented July 29, 1977

Typed by Mary Jo Stratton for Arthur Robert Anderson

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THIS THESIS IS DEDICATED TO

MY PARENTS

Agnes T. Anderson

Robert A. Anderson

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# TAXONOMY AND HOST SPECIFICITY OF THE GENUS *AGROBACTERIUM*

## INTRODUCTION

The crown-gall disease is worldwide in its distribution. The causal agent, *Agrobacterium tumefaciens* (Conn. 1942), generally requires a wound site to infect and induce a tumor. Although the bacterium can infect at least 140 plant species in 60 families (1), the most common natural hosts are pome and stone fruit trees, cane berries, grapes, and numerous ornamentals. Crown gall is a serious disease in Oregon nurseries, and depending upon environmental conditions, can reach epidemic proportions in any given year.

The disease is easily recognized by the presence of tumors or galls, of varying size and form, on the roots, shoots, stems, or crown of the plant. Although the host is generally not killed, plant vigor, size and/or yield can be greatly reduced. Consequently, diseased plants are unsalable and can result in great economic losses to the grower.

Another bacterium, *Agrobacterium radiobacter* (Conn. 1942), is indistinguishable from *A. tumefaciens* except for its non-pathogenic character. When agrobacteria were isolated from tumor tissue using semi-selective media, often only non-pathogenic isolates (as tested by inoculation to tomato) were recovered. Similarly, when soil from

heavily infected crown-gall fields was assayed, only non-pathogenic agrobacteria could be detected. The possibility of host specificity was raised to account for these observations.

Keane et al. (2) reported that there were two groups of agrobacteria in Australia, both of which contained pathogenic and non-pathogenic isolates. Therefore, the inability to detect pathogens in tumors and soil in the United States may have been due to overlooking one or more types of agrobacteria.

This study was undertaken to characterize United States *Agrobacterium* isolates, particularly from the Pacific Northwest, and to identify the degree of host specificity present among these isolates. Additionally, in the course of this investigation, efforts were made to find alternative methods for identifying isolate pathogenicity other than by inoculation to an assay host.

## Bibliography

1. Elliott, C. 1951. Agrobacterium tumefaciens, p. 6. In Manual of Bacterial Plant Pathogens. Chronica Botanica Co., Waltham, Mass.

2. Keane, P. J., A. Kerr and P. B. New. 1970. Crown gall on stone fruit. II Identification and nomenclature of Agrobacterium isolates. Aust. J. Biol. Sci. 23:585-595.

# I. CHARACTERIZATION OF AGROBACTERIUM ISOLATES FROM THE UNITED STATES AND A TAXONOMIC PROPOSAL

## INTRODUCTION

Species designations in the genus *Agrobacterium* are based solely on pathogenicity (1) and have generated considerable controversy and new taxonomic proposals (6, 13). Keane et al. (13) reported that all Australian *Agrobacterium* isolates tested could be differentiated by ten biochemical tests into two groups: biotypes 1 and 2. Kersters et al. (16) labeled these groups as clusters I and II, respectively, and White (30) as groups I and III, respectively. Both groups contained pathogenic and non-pathogenic isolates, and therefore they do not correspond to current taxonomy as defined by pathogenicity (1). Not all isolates conform to the group descriptions as several were shown to be intermediate in character (21). Although only a small number of intermediate isolates have been identified, Kersters et al. (16) suggested that a third group might be designated for these intermediate isolates.

Anderson and Moore (2) reported that *Agrobacterium* isolates unable to oxidize lactose to 3-ketolactose [K(-)] predominate in the Pacific Northwest. These isolates correspond to biotype 2 of Keane et al., cluster II of Kersters et al. and group III of White. Kerr (14) also reported that the same K(-) type predominates in Australia.

However, recent taxonomic studies from Europe and Australia have contained no more than 20 K(-) isolates and very few *A. radiobacter*, *A. rhizogenes* and *A. rubi* isolates. Therefore, it was desirable to compare the biochemical and physiological similarity of United States isolates to those found elsewhere in the world (13, 16, 21, 30).

## MATERIALS AND METHODS

### Cultures

Table 1 lists the cultures tested, their species designations, origin, and the supplier's name. The isolates from our laboratory were isolated from soil and naturally-occurring tumors present on 26 host species from 11 plant families (Table 2). Thirty-one, 30, and 39% of our isolates were selected from Schroth et al. (24), New and Kerr (20) and D1M (12) media. All cultures included in this study were cloned three times for purity to avoid mixed cultures as described by Wright et al. (31). Stock cultures were kept at 10 C in sterile distilled water blanks. Working cultures were grown on PDA (Difco) slants supplemented with 5.0 g $CaCO_3$/liter (1).

Twenty-six previously characterized Australian isolates (13) were included as comparative controls.

## Biochemical and Physiological Tests

Twenty selected tests were compared for their reported differentiating capabilities (cf. Table 4). Test media were incubated at 28 C unless stated otherwise. Liquid test media were inoculated with 0.1 ml of a cell suspension containing approximately $10^6$ colony forming units/ml. Slants were inoculated directly by streaking from the working cultures. Plates were replica plated (26) from a 24-36 hour old master plate. All isolate reactions were recorded after 14 days incubation, except liquid tube and slant media tests which were incubated for an additional week and then recorded. The individual test procedures are listed below.

1) 3-Ketolactose production (4).

2) Oxidase (13). Development of a purple coloration within 10 seconds was considered positive.

3) Litmus milk (Difco) reaction. Potentiometric measurement of the pH reaction also was measured.

4) Sodium chloride tolerance. Nutrient agar (Beef extract, 3.0 g/l; peptone, 5.0 g/l; and agar, 1.8%) supplemented with sodium chloride at a concentration of 1, 2, 3, 4, or 5% (w/v) was used.

5) Utilization of Malonate (17). A blue substrate coloration was positive.

6) Utilization of Citrate (25). A blue substrate coloration was positive.

7) Sodium selenite medium (10). The sodium selenite was added before autoclaving. Red coloration of the colony was positive.

8) Acid production from erythritol (9).

9) Growth on L-tyrosine (16).

10) Growth on sodium-D, L-lactate. The mineral basal medium of Kersters et al. (16) was used. A 1% (w/v) filter-sterilized solution of sodium-D, L-lactate and bromocresol purple (0.0016%, w/v) were added after autoclaving. Growth was measured as increased turbidity.

11) Growth on Schroth et al. medium (24).

12) Growth on New and Kerr medium (20).

13) Growth on D1M medium (12).

14) Growth on ferric ammonium citrate broth (10). Pellicle formation was positive.

15) Growth at pH 11.6. A mannitol (10.0 g/l)-yeast extract (5.0 g/l) medium (1.8% agar) was autoclaved and the pH adjusted to 11.6 with 40% (w/v) NaOH.

16) Maximum growth temperatures. Nutrient agar plates (see #4) were inoculated and incubated in thermostatically controlled, warm-air incubators at 25, 30, 34 and 40 C for up to two weeks.

17) Growth on glycerophosphate medium. Medium I of Riker et al. (22) was used. A white precipitate was positive.

18) Growth on raffinose medium (7).

19) Absorption of aniline blue (10).

20) Oxidation-reduction potential (8).

Pathogenicity of Isolates

The ability of all isolates to induce tumors on 12 host species was tested using previously described methods (3). Hairy root induction by A. rhizogenes was determined by the carrot assay (18).

# RESULTS

## Biochemical and Physiological Tests

Sixteen of the 20 biochemical and physiological tests used separated 72 United States (U.S.) and 26 Australian Agrobacterium isolates into two groups designated 3-ketolactose positive [K(+)] and 3-ketolactose negative [K(-)], similar to those described previously (13, 16, 21, 24). An additional 80 Agrobacterium isolates were tested further and similarly separated by these 16 diagnostic tests (Table 3). The remaining four tests (raffinose, aniline blue, D1M, and oxidation-reduction potential) gave extremely variable results and

failed to differentiate between the isolates. Others have reported similar difficulty with these four tests (10, 16).

Of the 16 diagnostic tests, isolate reactions to three tests (sodium selenite, maximum growth temperature, and glycerophosphate) differed from those previously reported (13, 21). Of the K(-) U.S. isolates, only 53% grew on the sodium selenite medium, whereas all 26 Australian isolates grew as previously reported (13). However, the K(+) U.S. isolates appeared light red to dark red in color, while the K(-) U.S. isolates that grew were white to slightly pink. The K(+) and K(-) groups could be differentiated subjectively by colony coloration. Therefore, when used simultaneously with other diagnostic tests, sodium selenite medium is helpful in separating the two strains.

The maximum growth temperatures for our isolates [K(+) = 40 C; K(-) = 34 C] were higher than reported by Panagopoulos and Psallidas (21) [K(+) = 37-38 C; K(-) = 29 C]. The reported temperature differences probably resulted from our use of warm-air, temperature-controlled incubators rather than stirred water baths as used by Panagopoulos and Psallidas. However the relative temperatures from both studies do agree and differentiate the two groups.

Only 71% of the U.S. K(-) isolates formed a white precipitate on glycerophosphate medium, whereas all 26 Australian isolates grew.

The specificity of this test was not as great in differentiating our U.S. and miscellaneous agrobacteria as Keane et al. (13) reported.

Isolate group reactions to sodium lactate and litmus milk were the same as reported (Table 4) even though alterations in methodology were made (cf. Materials and Methods). A 50% racemized mixture of sodium lactate gave the same test results as the D isomer (16). Since definitive isolate reactions in the litmus milk test were difficult to read via litmus color change, pH was measured potentiometrically and found to be more precise, especially for near-neutral pH reactions.

## Isolate Groups

One hundred seventy-eight *Agrobacterium* isolates of every recognized species were characterized in this study. Previously, the largest number of agrobacteria characterized in any report was 70 (16). Our isolates were biochemically and physiologically very similar to previously characterized isolates (13, 16, 21, 30). The 26 Australian isolates included as comparative controls gave test reactions identical to those reported by Keane et al. (13). Of the 120 United States (U.S.) and 32 isolates from other parts of the world, 136 formed two homogeneous groups which correspond closely with the groups identified by the 3-ketolactose test (Table 3). These two groups correspond to biotypes 1 and 2 of Keane et al. (13), clusters I

and II of Kersters et al. (16) and groups I and III of White (30), respectively. The remaining 16 isolates formed a heterogeneous group which had mixed reactions intermediate between the two homogeneous groups (Table 5). Since these intermediates represent a small proportion of the total number of naturally occurring agrobacteria, and disproportionally represents the total isolate reaction deviance, they are considered separately from the two homogeneous groups.

We favor designating the two groups 3-ketolactose positive [K(+)] and 3-ketolactose negative [K(-)] for several reasons. The 3-ketolactose test can be performed after incubating an isolate for only 24 hours and isolate reactions are easily discernible and very definitive. In addition, the ability to oxidize lactose to 3-ketolactose is a unique character of the genus <u>Agrobacterium</u> (4).

<u>The 3-Ketolactose Positive Group</u>. Of the isolates which oxidized lactose to 3-ketolactose, over 90% also were identified as K(+) by 15 of the 16 diagnostic tests except maximum growth temperature (Table 3). More accurately, 100% of all the K(+) isolates were identically grouped by seven of the 16 tests, indicating that the K(+) isolates form a very homogeneous group. All K(+) isolates were biochemically and physiologically indistinguishable from each other, regardless of pathological response as determined by Anderson and Moore (3).

<u>The 3-Ketolactose Negative Group</u>. The K(-) isolates also form a homogeneous group, but not as uniform as the K(+) group. Only nine of the 16 tests differentiated at least 90% of the K(-) isolates corresponding to the 3-ketolactose negative reaction (Table 2). Similarly, 100% of the K(-) isolates were identified by only two tests, ferric ammonium citrate and sodium chloride tolerance. Although five K(+) and 14 K(-) isolates were received as <u>A. rhizogenes</u>, only eight isolates, all K(-), induced hairy root formation (18). All K(-) isolates were biochemically and physiologically similar to one another, regardless of pathological response.

<u>Intermediate Isolates</u>. Sixteen isolates were identified which had test reactions intermediate between those of the two strains (Table 5). These isolates represent a small proportion of the total number of agrobacteria examined. The first 11 isolates listed in Table 5 were isolated in our laboratory and comprise only 9% of the total number of U.S. agrobacteria examined. Two, three, and six of these isolates were selected from Schroth et al., New and Kerr, and D1M media, respectively. Since Schroth et al. and New and Kerr roughly select for K(+) and K(-) isolates, respectively, and D1M is not strain specific, we can conclude that our isolation techniques did not bias the number of intermediates identified. Furthermore, since approximately one-third of all the U.S. isolates were selected from each of the three media types, we also can conclude that the

percentage of intermediates identified is representative of the number which occur naturally in the Pacific Northwest.

## DISCUSSION

Agreement between test results reported in this study and by others was good. The K(+) and K(-) U.S. isolates gave the same reactions as biotype 1 and 2 of Keane et al. (13) and groups I and III of White (30) for all common tests. However, the reactions of the U.S. isolates differed from those reported by Kersters et al. (16) for the oxidase, sodium selenite, and glycerophosphate tests and from the Greek isolates (21) for the oxidase, citrate, and raffinose tests. Two possible explanations for these test variations might be: i) differences in testing methods or media as discussed by Moustafa and Whittenbury (19) for the oxidase test; and/or ii) that geographical isolates of *Agrobacterium* differ in some of their biochemical and/or physiological characters as demonstrated when comparing Australian and U.S. isolate reactions to the sodium selenite and glycerophosphate tests.

All of the 16 intermediates characterized in this study were K(-) as were the seven intermediate isolates identified by Panagopoulos and Psallidas (21). They and Kersters et al. (16) suggested that a third group of agrobacteria may exist. I think this is unlikely since the number of intermediates is less than 10% of the total number of

agrobacteria examined. The homogeneity of both the K(+) and K(-) groups strongly suggests that the intermediate isolates are indeed only intermediates. For example, three isolates have nearly all but one K(+) character except for the 3-ketolactose reaction. This suggests that intermediate agrobacteria may arise when an isolate has lost the ability to produce 3-ketoglycosides. Furthermore, the loss of ability to form 3-ketoglycosides may be correlated with simultaneous loss of certain K(-) characteristics as suggested by Lippincott and Lippincott (18), since the majority of the intermediate isolate reactions to several tests (sodium chloride tolerance, citrate and erythritol) are K(-) in character while most other characters remained K(+).

The grouping of agrobacteria on the basis of biochemical and physiological tests which correlated with the 3-ketolactose reaction, forms a much stronger separation for speciation than pathogenicity. Pathogenic and non-pathogenic agrobacteria belonging to both the K(+) and K(-) groups were identified in this study. The presence of pathogenic isolates in both groups may be explained by the fact that pathogenicity in agrobacteria is associated with a large tumor-inducing (Ti) plasmid (5, 27, 28, 32). Transfer of the Ti plasmid to non-pathogenic agrobacteria (5, 15, 28, 29) or <u>Rhizobium trifolii</u> (11) results in the recipients acquiring the tumor-inducing ability. If pathogenicity can be transferred from pathogenic to non-pathogenic

agrobacteria in nature, then this character should be found among both the K(+) and K(-) strains, as indeed it is (13, 16, 21, 30). Pathogenicity, therefore, is not a stable isolate character and is of little taxonomic value. Furthermore, Anderson and Moore (3) found that all tumor-inducing agrobacteria examined were host-specific, indicating that host specificity also is inadequate for naming an *Agrobacterium* species, such as *A. rubi*. Currier and Nester (5) also reported that the K(+) isolates studied have a greater chromosomal homology to one another than to the K(-) isolates and vice versa. The two groups also seem to be serologically distinct (23).

Keane et al. (13) have proposed a new taxonomic classification for the genus *Agrobacterium*, wherein all agrobacteria are grouped under one species, pathogenicity is designated by a variety epithet and the two natural isolate groups are recognized by a biotype designation. I agree that pathogenicity is an important *Agrobacterium* character, but should not be recognized as a variety epithet since it is an easily transferred isolate character. I believe that the differences between the K(+) and K(-) strains of agrobacteria have been well documented and are of such magnitude to be recognized at the species level. In addition, the proposed species names of Keane et al. are lengthy and cumbersome to use.

I propose that the currently recognized species names *Agrobacterium tumefaciens* be designated for the K(+) group and that

Agrobacterium rhizogenes be designated for the K(-) group. This proposal is made, knowing that the name "radiobacter" has historical preference over "rhizogenes" according to the International Code of Nomenclature of Bacteria and Viruses (1958), for the following reasons: i) traditionally the name "radiobacter" has been synonymous with non-pathogenic. Since many characterized K(-) isolates are pathogenic, "radiobacter" would be misleading. ii) The name "rhizogenes" has traditionally signified pathogenicity in the genus Agrobacterium. Furthermore, it has been associated with the K(-) strain, since all confirmed hairy root isolates are 3-ketolactose negative. Since the type or lack of pathological response exhibited by the host-pathogen complex is of great practical importance, the letters "T" (tumorigenic), "HR" (hairy root) and "AV" (avirulent) could be placed after the species name to identify a specific isolate-host interaction. Those isolates found intermediate in their reactions to the two groups would not be given a specific epithet, but would be identified as Agrobacterium spp. until more strains are obtained and further characterized. I believe that the proposed taxonomic scheme outlined below better reflects the natural relationships present among Agrobacterium isolates and that it is a simple and practical system to use since species designations can be easily assigned with a minimum amount of effort.

Agrobacterium tumefaciens T, AV
(3-ketolactose positive)

Agrobacterium rhizogenes T, HR, AV
(3-ketolactose negative)

## SUMMARY

One hundred seventy-eight Agrobacterium isolates (120 United States (U.S.), 26 Australian, and 32 of miscellaneous origin) were characterized using 20 biochemical and physiological tests. Sixteen tests separated over 90% of the isolates into two homogeneous groups designated the 3-ketolactose positive strain [K(+)] and the 3-ketolactose negative strain [K(-)]. The U.S. isolates are quite similar, but not identical in character to the 26 Australian isolates. Sixteen isolates, all K(-), did not conform to either strain description. It is improbable that these isolates form a third strain of agrobacteria, since they form a very heterogeneous group with each isolate being unlike the rest. The relationship between the K(+) and K(-) strains is discussed. Isolates of the species A. tumefaciens and A. rubi were biochemically and physiologically indistinguishable. All confirmed A. rhizogenes isolates were K(-) in character. A taxonomic proposal for the genus Agrobacterium is presented, wherein two species would be recognized: Agrobacterium tumefaciens for the K(+) group and Agrobacterium rhizogenes for the K(-) group with appropriate designations to indicate pathogenicity.

Table 1. Agrobacterium species and isolates used and their origins.

| Species & isolates | 3-Ketolactose reaction | Source[a] | Isolated from[b]: |
|---|---|---|---|
| Agrobacterium tumefaciens | | | |
| AB11/73 | + | L.W. Moore | Lippia canescum |
| AB12/73 | + | " | L. canescum |
| C5/73 | + | " | Sorbus sp. |
| E9/73 | + | " | Dahlia sp. |
| G1/73 | + | " | Prunus persica |
| H7/73 | + | " | Malus sp. |
| K15/73 | + | " | Salix sp. |
| N2/73 | + | " | Raspberry |
| RR9 | + | " | Rubus idaeus |
| S1/73 | + | " | Lippia canescum |
| S2/73 | + | " | L. canescum |
| S7/73 | + | " | L. canescum |
| T28/73 | + | " | Rosa sp. |
| A25/75 | + | " | Prunus avium |
| CG-1C | + | " | Pear |
| A-13333 | + | D. Huisingh | Labeled as A. rhizogenes but does not form hairy root |
| 30 | + | A. Kerr | Peach |
| A6-NC | + | E.W. Nester | Nonclumping derivative (Nester) of A6 received - R. Schilperoort |
| $B_2A$ | + | " | INA |
| B6-806 | + | " | Phage PS8 sensitive derivative of B6 |
| IIBV7 | + | " | Subculture of IIB from Chrysanthemum frutescens |
| 15955 | + | " | INA |
| T37 | + | " | Walnut |
| A136 | + | " | INA |
| B6 | + | R. Baker | Apple seedling |
| 16 | + | Unknown | |
| 26 | + | " | |
| C58a | + | " | |
| AT1a | + | " | |
| AB1/73 | - | L.W. Moore | Lippia canescum |
| AB2/73 | - | " | L. canescum |

(Continued on next page)

Table 1 (Continued)

| Species & isolates | 3-Ketolactose reaction | Source[a] | Isolated from[b]: |
|---|---|---|---|
| AR5K/71 | - | L. W. Moore | *Malus* sp. |
| B3/73 | - | " | *Acer platanoides* |
| B4/73 | - | " | *A. platanoides* |
| E8/73 | - | " | *Dahlia* sp. |
| K6/73 | - | " | *Salix* sp. |
| K9/73 | - | " | *Salix* sp. |
| M2/73 | - | " | *Betula* sp. |
| M3/73 | - | " | *Betula* sp. |
| N4/73 | - | " | Raspberry |
| T3/73 | - | " | *Rosa* sp. |
| T10/73 | - | " | *Rosa* sp. |
| U-11 | - | " | *Salix* sp. |
| W1/73 | - | " | *Euonymus* sp. |
| W2/73 | - | " | *Euonymus* sp. |
| B1/74 | - | " | *Prunus tomentosa* |
| B2/74 | - | " | *P. tomentosa* |
| C2/74 | - | " | *P. avium* |
| C3/74 | - | " | *P. avium* |
| I1/75 | - | " | *Rosa* sp. |
| I7/75 | - | " | *Rosa* sp. |
| I10/75 | - | " | *Rosa* sp. |
| I15/75 | - | " | *Rosa* sp. |
| I16/75 | - | " | *Rosa* sp. |
| A21/75 | - | " | *Prunus avium* |
| 32 | - | A. Kerr | Almond |
| 34 | - | " | Peach |
| 35 | - | " | Peach |
| 36 | - | " | Peach |
| 37 | - | " | Peach |
| 39 | - | " | Plum |
| 40 | - | " | Plum |
| 41 | - | " | Almond |
| K27 | - | " | Peach gall |
| 727 | - | PDD | INA |
| 3667 | - | PDD | INA |
| B234 | - | DeVay | INA |
| 0362 | - | E. W. Nester | Soil |
| 6467 | - | " | Soil |
| At181a | - | Unknown | |
| EU-6a | - | " | |

(Continued on next page)

Table 1 (Continued)

| Species & isolates | 3-Ketolactose reaction | Source[a] | Isolated from[b]: |
|---|---|---|---|
| TT133a | - | Unknown | |
| 223a | - | " | |
| 21a | - | " | |
| Agrobacterium radiobacter | | | |
| B6/73 | + | L.W. Moore | Acer platanoides |
| B7/73 | + | " | A. platanoides |
| C1/73 | + | " | Sorbus sp. |
| D22/73 | + | " | Prunus avium |
| D42/73 | + | " | P. avium |
| E13/73 | + | " | Dahlia sp. |
| F2/73 | + | " | Rosa sp. |
| F5/73 | + | " | Rosa sp. |
| F9/73 | + | " | Rosa sp. |
| G12/73 | + | " | Prunus persica |
| G13/73 | + | " | P. persica |
| Ga7/72 | + | " | Soil |
| H2/72 | + | " | Azalea sp. |
| H9/73 | + | " | Malus sp. |
| J8/73 | + | " | Dahlia sp. |
| K12/73 | + | " | Salix sp. |
| K13/73 | + | " | Salix sp. |
| L11/73 | + | " | Soil |
| L13/73 | + | " | Soil |
| L51/73 | + | " | Prunus avium |
| L180/73 | + | " | Inoculated soil |
| N6/73 | + | " | Rubus sp. |
| N7/73 | + | " | Rubus sp. |
| P10/73 | + | " | Gypsophila sp. |
| S9/73 | + | " | Lippia canescum |
| T20/73 | + | " | Rosa sp. |
| B1/75 | + | " | Malus domestica 'Malling' |
| B4/75 | + | " | M. domestica 'Malling' |
| B7/75 | + | " | M. domestica 'Malling" |
| B8/75 | + | " | M. domestica 'Malling" |
| 1 | + | A. Kerr | Prunus cerasifera |
| 3 | + | " | Peach |
| 4 | + | " | Pear |
| 5 | + | " | Soil |
| 6 | + | " | Soil |

(Continued on next page)

Table 1 (Continued)

| Species & isolates | 3-Ketolactose reaction | Source[a] | Isolated from[b]: |
|---|---|---|---|
| 12-XI-28 | + | L.W. Moore | Soil from around hops (Oregon, USA) |
| 14-XIV-6 | + | " | Soil from around beans (Oregon, USA) |
| 18-XVI-50 | + | " | Soil from around garlic (Oregon, USA) |
| AT4 | + | E.W. Nester | Dahlia rabitz |
| B6-T | + | " | INA |
| RV3 | + | " | INA |
| TR1 | + | " | INA |
| A216 | + | " | Bacteriocin 84 resistant derivative of T37 |
| 15a | + | Unknown | |
| 27a | + | " | |
| AA1/73 | - | L.W. Moore | Libocedrus decurrens |
| AA10/73 | - | " | L. decurrens |
| AR4K/71 | - | " | Malus sp. |
| D4/73 | - | " | Prunus avium |
| D16/73 | - | " | P. avium |
| E3/73 | - | " | Dahlia |
| G6/73 | - | " | Prunus persica |
| H4/73 | - | " | Malus sp. |
| H7/72 | - | " | Malus sp. |
| L20/73 | - | " | Prunus cerasifera |
| L23/73 | - | " | P. cerasifera |
| L42/73 | - | " | Prunus avium |
| L47/73 | - | " | P. avium |
| L241/73 | - | " | Inoculated soil |
| P6/73 | - | " | Gypsophila sp. |
| P8/73 | - | " | Gypsophila sp. |
| S5/72 | - | " | Libocedrus decurrens |
| S11/72 | - | " | L. decurrens |
| B6/74 | - | " | Prunus besseyi |
| B7/74 | - | " | P. besseyi |
| B11/74 | - | " | Prunus tomentosa |
| B12/74 | - | " | P. tomentosa |
| B13/74 | - | " | Prunus besseyi |
| B14/74 | - | " | P. besseyi |
| D1/74 | - | " | Photinia sp. |
| D5/74 | - | " | Photinia sp. |

(Continued on next page)

Table 1 (Continued)

| Species & isolates | 3-Ketolactose reaction | Source[a] | Isolated from[b]: |
|---|---|---|---|
| F3/74 | - | L.W. Moore | Cucumis sativus |
| F4/74 | - | " | C. sativus |
| I8/75 | - | " | Rosa sp. |
| B10/75 | - | " | Malus domestica 'Malling' |
| B12/75 | - | " | M. domestica 'Malling' |
| AA17/73 | - | " | Libocedrus decurrens |
| I2/73 | - | " | Rosa sp. |
| 84 | - | A. Kerr | Soil around peach gall |
| 725 | - | PDD | INA |
| Agrobacterium rhizogenes | | | |
| 45 | - | A. Kerr | Apple |
| 46 | - | " | INA |
| 47 | - | " | INA |
| 48 | - | " | INA |
| 68 | - | " | INA |
| 25818 | - | ATCC | Apple (Kerr 45) |
| A-2 | - | D. Huisingh | INA |
| A-3 | - | " | INA |
| A-4 | - | " | INA |
| A-8 | - | " | INA |
| 15834 | - | E.W. Nester | INA |
| 11325I | - | " | INA |
| 11325II | - | " | INA |
| A-1 | + | D. Huisingh | INA |
| A-28 | + | " | INA |
| TR102 | + | E.W. Nester | Apple (ATCC 13332) |
| TR104 | + | " | INA (ATTC 13333) |
| 49a | + | Unknown | |
| TR7a | - | " | |
| Agrobacterium rubi | | | |
| RR5 | + | L.W. Moore | Rubus idaeus |
| TR2 | - | E.W. Nester | Probably black raspberry (ATCC 13334) |
| 13335 | - | " | Probably boysenberry |

[a]PPD = Plant Disease Division Culture Collection, Auckland, N.Z.
ATCC = American Type Culture Collection

[b]INA = Information Not Available

Table 2. Plant sources of United States isolates.

| Family | Genus | Common name |
|---|---|---|
| Aceraceae | *Acer platanoides* | Norway maple |
| Betulaceae | *Betula* sp. | Birch |
| Caryopyyllaceae | *Gypsophila* sp. | Baby's breath |
| Celastraceae | *Euonymus fortunei* | Wahoo |
| Compositae | *Dahlia* sp. | Dahlia |
| Cucurbitaceae | *Cucumis sativus* | Cucumber |
| Cupressaceae | *Libocedrus decurrens* | Incense cedar |
| Ericaceae | *Azalea* sp. | Azalea |
| Rosaceae | *Malus domestica* | Malling apple |
| | *Malus* sp. | Crab apple |
| | *Photinia* sp. | Photinia |
| | *Rosa* sp. | Rose |
| | *Prunus amygdalus* | Almond |
| | *P. avium* | Mazzard cherry |
| | *P. besseyi* | Besseyi cherry |
| | *P. cerasifera* | Myrobalan plum |
| | *P. domestica* | Wild plum |
| | *P. mahaleb* | Mahaleb cherry |
| | *P. persica* | Peach |
| | *P. tomentosa* | Tomentosa cherry |
| | *Pyrus communis* | Pear |
| | *Rubus idaeus* | Red raspberry |
| | *R. ursinus* | Boysenberry |
| | *Sorbus* sp. | Mountain ash |
| Salicaceae | *Salix* sp. | Willow |
| Verbenaceae | *Lippia canescum* | Lippia |

Table 3. Characterization of 136 Agrobacterium isolates for reaction to 16 biochemical tests. Results are given as percentage of isolates with the 3-ketolactose positive [K(+)] strain or negative [K(-)] strain reaction.

| Biological and physiological tests | K(+)[a] | %[b] | K(-)[c] | %[d] |
|---|---|---|---|---|
| 3-Ketolactose | + | 100 | - | 100 |
| Ferric ammonium citrate | + | 100 | - | 100 |
| NaCl tolerance | 3-4% | 98.6 | 1% | 100 |
| L-tyrosine | - | 100 | + | 93.8 |
| Schroth et al. | + | 97.1 | - | 98.5 |
| Erythritol | - | 98.5 | + | 95.3 |
| New and Kerr | - | 98.5 | + | 92.4 |
| Oxidase | + | 98.6 | - | 92.2 |
| Citrate | - | 97.0 | + | 92.3 |
| Na-D, L-lactate | + | 100 | - | 84.8 |
| Malonate | - | 100 | + | 83.1 |
| pH 11.6 | + | 97.1 | - | 86.7 |
| Litmus milk | b | 100 | a | 76.6 |
| Na selenite | + | 100 | - | 74.2 |
| Glycerophosphate | + | 100 | - | 70.6 |
| Maximum growth temperature | 40 C | 84.8 | 34 C | 90.3 |

[a]K(+) strain reactions

[b]Percentage of isolates positive for the 3-ketolactose test which have the test reactions for a given test corresponding to the K(+) strain reactions.

[c]K(-) strain reactions

[d]Percentage of isolates negative for the 3-ketolactose test which have the test reactions for a given test corresponding to the K(-) strain reactions.

Table 4. Comparison of diagnostic tests results for the 3-ketolactose positive and negative *Agrobacterium* strains as reported in several recent taxonomic references.

| Diagnostic tests | Strain reactions[a] (US isolates) K(+)[b] | K(-)[b] | Literature references with reported biochemical and physiological test results: Keane et al. (13) | Kersters et al. (16) | Panagopoulos and Psallidas (21) | White (30) |
|---|---|---|---|---|---|---|
| 3-Ketolactose | + | - | Same results | Same results | Same results | Same results |
| Oxidase | + | - | Same results | Variable | All positive | Not tested |
| Litmus milk | b | a | Same results | Same results | Same results | Same results |
| NaCl tolerance | 3-4% | 1% | Not tested | Not tested | Same results | Same results |
| Citrate | - | + | Same results | Same results | All positive | Not tested |
| Erythritol | - | + | Same results | Not tested | Same results | Same results |
| Na selenite | + | - | Same results | Variable | Not tested | Not tested |
| L-tyrosine | - | + | Not tested | Same results | Not tested | Not tested |
| Na-D, L-lactate | + | - | Not tested | Same results[c] | Not tested | Not tested |
| Schroth et al. | + | - | Not tested | Not tested | Not tested | Not tested |
| New and Kerr | - | + | Not tested | Not tested | Not tested | Not tested |
| Malonate | - | + | Same results | Not tested | Same results | Same results |
| Ferric ammonium citrate | + | - | Same results | Same results | Not tested | Not tested |
| pH 11.6 | + | - | Not tested | Not tested | Not tested | Not tested |
| Glycerophosphate | + | - | Same results | Not tested[d] | Not tested | Not tested |
| Maximum growth temp. | 40 C | 34 C | Not tested | Not tested | Similar results[e] | Similar results[e] |
| Raffinose | V | V | Not tested | Not tested | K(+), +; K(-), - | Not tested |
| Analine blue | V | V | Same results | Same results | Not tested | Not tested |
| Oxidation-reduction | V | V | Same results | Not tested | Not tested | Not tested |
| DIM | V | V | Not tested | Not tested | Not tested | Not tested |

[a]Abbreviations for the test reactions are: + = positive; - = negative; b = alkaline; a = acid; and V = variable.

[b]K(+) = 3-ketolactose positive strain and K(-) = 3-ketolactose negative strain.

[c]Only the D isomer was tested.

[d]A white precipitate was not tested for on glycerophosphate medium.

[e]The relative temperatures between the studies were the same, but the absolute temperatures were not.

Table 5. Biochemical and physiological reactions[a] of 16 intermediate Agrobacterium isolates to 14 tests.

| Isolate | Source | Pathogenicity[b] | 3-Ketolactose | Oxidase | Litmus milk | NaCl tolerance | Citrate | Erythritol | Na selenite | L-tyrosine | Na-D, L-lactate | Schroth et al. | New and Kerr | Malonate | Ferric ammonium citrate | pH 11.6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H7/72 | Azalea | - | - | - | a | 5 | - | + | + | + | + | - | + | - | + | + |
| E3/73 | Dahlia | - | - | + | b | 4 | + | + | + | + | + | + | + | - | + | - |
| L47/73 | Cherry, Mazzard | - | - | + | b | 1 | + | - | + | + | + | - | - | + | - | - |
| L180/73 | Soil | - | - | - | a | 5 | - | + | + | + | + | - | - | - | + | + |
| L241/73 | Soil | - | - | + | b | 1 | + | + | + | - | + | - | - | - | - | - |
| W1/73 | Enonymus | + | - | + | b | 1 | + | - | + | - | - | - | - | + | - | + |
| W2/73 | Enonymus | + | - | + | b | 1 | + | - | + | - | - | - | - | + | - | - |
| P6/73 | Baby's breath | - | - | + | b | 5 | - | + | NT | - | + | + | - | - | - | NT |
| P8/73 | Baby's breath | - | - | + | b | 1 | + | + | NT | + | + | + | + | - | + | NT |
| B10/75 | Apple, Malling | - | - | + | b | 1 | - | + | NT | + | | + | + | - | + | NT |
| B12/75 | Apple, Malling | - | - | + | b | 1 | - | + | NT | + | - | + | + | - | + | NT |
| AT181a | | + | - | + | b | 4 | - | - | NT | - | + | + | - | - | + | NT |
| 0362 | | + | - | + | b | 5 | - | + | NT | - | + | + | - | - | + | NT |
| 11325 I | | + | - | + | b | 1 | + | - | NT | - | - | - | - | + | - | NT |
| 11325 II | | + | - | + | b | 1 | + | - | NT | - | - | - | - | + | - | NT |
| 13335 | | + | - | + | b | 3 | + | - | NT | - | + | - | - | + | - | NT |
| | | | | | Characteristic reactions for the two strains of Agrobacterium | | | | | | | | | | | |
| K(+)[c] | | | + | + | b | 3-4% | - | - | + | - | + | + | - | - | + | + |
| K(-)[c] | | | - | - | a | 1% | + | + | - | + | - | - | + | + | - | - |

[a]Abbreviations for the test reactions are: + = positive; - = negative; b = alkaline; a = acid; and NT = not tested.

[b]Pathogenicity was tested on Bonny Best tomatoes.

[c]K(+) and K(-) denote the two strains of agrobacteria, one 3-ketolactose positive and the other 3-ketolactose negative. The characteristic test reactions of each strain are given.

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# II. HOST SPECIFICITY IN THE GENUS *AGROBACTERIUM*

## INTRODUCTION

Speciation in the genus *Agrobacterium* is based upon pathogenicity (1), usually determined on 1-3 host plants (16). For example, the species *Agrobacterium rubi* is currently defined by its ability to induce tumors on *Rubus* spp. (1). Despite the knowledge that host specificity exists among *Agrobacterium tumefaciens* isolates (11, 17, 18, 19, 21), no one has studied the range of its occurrence among naturally occurring agrobacteria. If host specificity is common among agrobacteria, then the host range should vary among isolates and the potential for incorrect identification of an isolate's pathogenic character is considerable. Lippincott and Lippincott (16) have recently questioned the validity of current species designations based upon testing only 1-3 host plants. The objectives were to determine the degree of host specificity and host range of a large number of isolates. The results are discussed relative to current species designations and the role of plasmids in tumorigenesis.

## MATERIALS AND METHODS

### Cultures and Inocula

One hundred seventy-eight previously characterized *Agrobacterium* isolates (2) were inoculated to a total of 11 plant species,

except as noted. Inocula were grown on potato dextrose agar (Difco) slants supplemented with 5.0 g $CaCO_3$/liter.

## Growth and Inoculation of Plants

All inoculation experiments were repeated 2-3 times using 1-3 plants of each species per isolate. Plants were grown under artificial lights (16 hour photoperiod) in either a greenhouse or growth chamber at 25 C day and 21 C night. Plants were inoculated by wounding herbaceous stems and leaves with multiple needle wounds and woody stems with a single scalpel cut. A loopful of inoculum from the slants was immediately applied to the wound with a sterile loop. Tumor formation on herbaceous plants was recorded after 4 weeks and after 8 weeks on woody plants. Small growths on datura were recorded as negative after 4 weeks incubation because datura has been reported to form outgrowths in response to wounding which are very similar to small tumors (7). It also was noted that outgrowths on datura ceased enlargement after 2-3 weeks of growth, while bacteria-induced tumors were not self-limiting.

Hairy root (HR) induction was tested by the method of Lippincott and Lippincott (15) using carrot (*Daucus carota*) discs.

## Host Plants Tested

The following plants were inoculated with all isolates: sugar

beet (*Beta vulgaris*) monogerm variety; daisy (*Chrysanthemum frutescens*) 'Paris'; datura (*Datura stramonium*); sunflower (*Helianthus annus*); bryophyllum (*Kalanchoe daigremontiana*); pea (*Lathyrus hirsutus*) 'Austrain'; tomato (*Lycopersicon esculentum*) 'Bonny Best'; green bean (*Phaseolus vulgaris*) 'Spartan Arrow'; Myrobalan plum (*Prunus cerasifera*); and radish (*Raphanus sativus*) 'Early Scarlet Globe'.

A second group of plant species was inoculated with selected non-pathogenic *Agrobacterium* isolates to identify pathogenicity on either i) additional host species, or ii) the homologous host (the plant from which an isolate was originally isolated). The numbers in parentheses indicate the number of isolates tested on a given host; cucumber (*Cucumis sativus*) 'Chicago Pickling' (25); incense cedar (*Libocedrus decurrens*) (3); lippia (*Lippia canescum*) (35); geranium (*Pelargonium* hybrid) 'Snowball' (102); apple (*Malus domestica*) open-pollinated 'Rome' seedlings (179); red raspberry (*Rubus idaeus*) (7); boysenberry (*Rubus ursinus* var. *loganobaccus boysen*) 'Thornless Young' (7); and zinnia (*Zinnia elegans*) 'Envy' (27).

## RESULTS

Host specificity was demonstrated by all 92 pathogenic *Agrobacterium* isolates. None of the isolates were pathogenic on every plant species (Figure 1 and Table 1). The patterns of infection of all

isolates on the 11 host species appeared randomly distributed as demonstrated by the isolates listed in Table 2. These isolates indicate that no common group of plant species was infected by all pathogenic isolates. Furthermore, the host range of an isolate was not apparently influenced by the plant species of isolation. Eighteen isolates obtained from 18 diverse naturally infected host species all had different host ranges, infecting from one to ten plant species. In addition, isolates obtained from different tumors on the same host species, or even from the same tumor, had variable host ranges resulting in diverse host specificity patterns (Table 3). Isolates AB1/73, AB2/73, AB11/73, and AB12/73 were all from the same lippia tumor. Similarly, isolates S1/73, S2/73, and S9/73 were isolated from a single lippia tumor. Therefore, isolates of the same origin can differ in their host specificity and host ranges.

The agrobacteria examined could be grouped only by the total number (but not kind) of hosts infected (Figure 1). Of 86 pathogenic agrobacteria, 59 infected six to eight of the 11 host species. Two isolates, M2/73 isolated from birch and N2/73 isolated from red raspberry, exhibited a wider host range, infecting nine and ten of 11 hosts, respectively. The remaining 25 isolates induced tumors on five or fewer hosts.

Twenty-seven of the 85 isolates non-pathogenic on the 11 hosts used were screened further for pathogenicity on lippia, cucumber and

zinnia. None of the isolates infected these additional host species. However, three of the non-pathogenic isolates were identified as pathogenic when inoculated to their homologous host (the plant species from which an isolate was originally isolated). Interestingly, inoculation of an isolate to its homologous host cannot assure correct pathological identification, as shown by isolates AB1/73 and AB11/73 (Table 2). These isolates were not pathogenic on lippia (homologous host), but did infect a total of 2 and 5 other plant species, respectively.

Each of the three _A. rubi_ isolates was pathogenic on at least seven hosts other than _Rubus_ spp.; conversely, several _A. tumefaciens_ isolates were also pathogenic on _Rubus_ spp. No morphological differences in the tumors incited by _A. rubi_ and _A. tumefaciens_ were noted on any of the hosts.

Of 19 isolates received as _A. rhizogenes_ from other laboratories and the American Type Culture Collection, only eight formed hairy root (HR) on carrot discs. Five of the eight confirmed HR cultures also formed tumors on at least four of the 11 hosts tested. Lippincott and Lippincott (15) similarly reported that of six _A. rhizogenes_ isolates examined, all formed HR on carrot discs and induced tumors on pinto beans. Even though each isolate was cloned via single-colony isolation three times on solid agar media before testing, those isolates causing tumors and HR formation may have been mixed as described by Wright et al. (24).

The efficacy of each plant species to indicate the pathogenic character of agrobacteria was evaluated (Table 1). Seventy-two of the 89 isolates were pathogenic on tomato and datura, while 70 and 69 isolates were pathogenic on sunflower and bryophyllum, respectively. However, only eight of the 17 isolates non-pathogenic on tomato were also non-pathogenic on datura, showing that not all isolates that infect tomato will infect datura and vice versa. If tomato and datura are used in combination as assay hosts, 90% of the pathogenic isolates were identified, versus 80% when either host species was used singularly (Figure 2). Combinations of tomato, datura, sunflower and bryophyllum could increase the number of pathogenic agrobacteria detected, but by no more than 3%.

## DISCUSSION

The genus Agrobacterium is noted for its broad host range (1, 5) and researchers have been inclined to view individual isolates or strains in a similar manner. This study shows that for individual pathogens host specificity is the rule and not the exception; however, as a group our isolates infected a continuum of from one to at least ten host species. The majority (66%) of these pathogens infected randomly, a total of six to eight of 11 plant species tested. The number of isolates which infected less than six or greater than eight

host plants dropped sharply, indicating that some factor may function to limit the number of species which an isolate can infect.

This factor may be associated with the Ti plasmid because preliminary data of Moore and Allen (unpublished) showed that an *in planta* cross between a host-specific 3-ketolactose negative [K(-)] pathogen and A136 (a 3-ketolactose positive [K(+)] mutant of NT-1, resistant to rifampicin and nalidixic acid, E.W. Nester) produced K(+) exconjugants which were pathogenic only on the same host as the donor. In addition to the probability that the genes for host specificity are located on the Ti plasmid, expression of these genes may be modified in some host species and not in others, resulting in a more restricted host range.

Further evidence for modified gene expression resulting in host specificity is the very different host specificity patterns observed for isolates TR104 and A13333. Both isolates are listed by the *American Type Culture Collection Catalogue* (22) as subcultures of Dr. A. J. Riker's *Agrobacterium rhizogenes* isolate (18), but TR104 forms tumors on bryophyllum, daisy, sunflower and datura, while A13333 induces tumors only on daisy. Neither, however, induce hairy root (HR) on carrot discs, as earlier reported (10, 15). Biochemically and physiologically, both isolates are 3-ketolactose positive [K(+)] and have all the K(+) group characteristics except for the inability to grow on Schroth et al. medium (2). This inability is a very unique

character since of 80 K(+) isolates tested, only TR104 and A13333 were unable to grow on Schroth et al. medium. This unique character suggests that both are true subcultures of Riker's A. rhizogenes isolate even though neither form HR on carrot. The narrower host range of A13333 would suggest that some genes regulating host specificity have been lost or modified with culturing, not unlike the loss of pathogenicity reported with continued culturing of some species of phytopathogenic bacteria. I have observed similar changes in the host ranges of double-drug resistant agrobacteria in inoculated field soil (Anderson and Moore, unpublished data).

Those factors responsible for modified host specificity may function at any point in the tumor induction process. Cell-site attachment and bacterial metabolism at the wound site are two known steps required for tumorigenesis (16). Auxin, produced by both agrobacteria and plants, is believed to be essential for at least one stage of tumor induction (12). Although the host is believed to be the primary source of the required growth hormones, several isolates have been reported (3, 13) which require exogenous auxin to induce tumors on some hosts species, but not on others. This suggests that auxin may be one factor responsible for host specificity among agrobacteria. Similarly, the nutritional status of an isolate in different host species also may be implicated, since bacterial metabolism is reportedly required in the induction process (14).

The observed host specificity among all agrobacteria studied implies that speciation in Agrobacterium based solely upon pathogenicity is of little taxonomic value. A. rubi and A. tumefaciens isolates infected the same plant species, including Rubus spp., and therefore are pathologically identical. McKeen has reported similar results (17). Anderson and Moore (2) and others (4, 6, 9, 23) using biochemical and physiological tests, also have concluded that A. rubi is indistinguishable from A. tumefaciens. Furthermore, any isolate may be host specific for a single host species; therefore, speciation based upon host specificity is unjustifiable. A new taxonomic proposal has been submitted which incorporates these observations (2).

Pathogenicity and host specificity also may be inadequate for defining the HR isolates. Five of eight confirmed A. rhizogenes isolates were found to form tumors on at least four of 11 plant species. Lippincott and Lippincott (15) have reported the same results. If HR isolates are host specific for their tumor-inducing ability, then they would logically be host specific for hairy root induction as well. Elevation of a Ti isolate to a new species because of HR inducing ability seems to be impractical, if not unjustified.

The problem of identifying HR isolates may be due in part to the pathological assay methods used. The carrot assay (15) is the most widely accepted and employed method for identifying A. rhizogenes. If isolates are host specific for HR production on natural host plants,

then the carrot assay may bias our identification of A. rhizogenes isolates. Interestingly, all our HR isolates first induced tumors followed by HR formation when inoculated to carrot discs. The genes for HR formation may be similar to or identical with the tumor-inducing genes on the Ti plasmid. Their pathological expression may be modified by other gene products coded for on the plasmid or chromosome, such as cytokinin production (8).

An auxiliary part of this study was to identify those plant species which best served as assay hosts for identifying pathogenic agrobacteria. Although the economics of time and space must be considered in addition to plant response, we make the following recommendations when assaying for pathogenicity. i) Two or more plants of any one species should be inoculated with each isolate. Occasionally we have observed that only one of two identically inoculated plants will form a tumor. ii) One or more of the following plant species should be used as assay hosts: tomato, datura, sunflower, or bryophyllum. Using combinations of these species, the pathogenic character of an isolate was identified with 78-94% certainty. iii) Since isolates may be very host specific, isolates not pathogenic on the assay host(s) used should be inoculated to the homologous plant species.

Using isolates with varied host specificity patterns as tools in genetic and ecological studies should facilitate our understanding of

host-specific mechanism(s) in agrobacteria. Studies of this nature are presently being conducted.

## SUMMARY

One hundred seventy-eight *Agrobacterium* isolates, principally of United States (U.S.) origin, were inoculated to 11 known crown-gall hosts to determine their host range. The U.S. isolates were isolated from 26 host species in 11 families. No isolate was pathogenic on all hosts tested. The host specificity of all isolates varied greatly since no host was infected by more than 81% of the pathogenic isolates. Sixty-six percent of the pathogenic isolates infected six to eight of 11 hosts tested while 3% infected only the host plant from which they were originally isolated (homologous host). However, the host range of an isolate is not influenced by the plant of isolation. Twenty-seven isolates (non-pathogenic on the initial 11 hosts tested) remained non-pathogenic when inoculated to an additional three host species. *A. rubi* isolates were pathologically indistinguishable from *A. tumefaciens* isolates. Five of eight confirmed *A. rhizogenes* isolates formed tumors in addition to hairy root. Evidence for change in an isolate's host specificity pattern in culture is presented and discussed relative to its relationship with the Ti plasmid. Speciation based upon pathogenicity and host specificity was of little taxonomic or practical value.

At present, non-characterized *Agrobacterium* isolates must be assayed for pathogenicity on a living host plant. Tomato and datura were infected by more of the pathogenic agrobacteria (81% each) than any other host species, thus serving as the best indicators of an isolate's pathogenicity of the 11 hosts tested.

Table 1. The percentage of pathogenic *Agrobacterium* isolates identified as tumor-inducing on each of 12 host plants tested.

| Common name of hosts | Number of pathogenic isolates inducing tumors[a] | Percentage of pathogenic isolates identified by each host plant |
|---|---|---|
| Tomato | 72 | 80.9 |
| Datura | 72 | 80.9 |
| Sunflower | 70 | 78.7 |
| Bryophyllum | 69 | 77.5 |
| Pea | 65 | 73.0 |
| Radish | 55 | 61.8 |
| Geranium | 27 | 50.0 |
| Plum | 43 | 48.3 |
| Bean | 33 | 37.1 |
| Daisy | 23 | 25.8 |
| Sugar beet | 6 | 6.7 |
| Apple | 2 | 2.2 |

[a]Eighty-nine pathogenic *Agrobacterium* isolates were tested on each host plant except on geranium (54 isolates).

Table 2. Host specificity patterns of 18 Agrobacterium isolates, isolated from 18 different naturally infected plants, as determined on 11 assay hosts.

| Isolates | Host species of isolation | Common names of 11 host species tested | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Apple | Bean | Beet | Bryophyllum | Plum | Daisy | Datura | Pea | Radish | Sunflower | Tomato |
| W2/73 | Euonymus | - | + | - | + | - | - | + | - | + | + | + |
| K6/73 | Willow | - | - | - | + | + | - | + | + | + | + | + |
| AR5K/71 | Apple | - | - | - | - | - | - | - | - | - | - | + |
| 30 | Peach | - | - | - | + | - | - | - | - | - | - | + |
| T3/73 | Rose | - | - | - | + | - | - | + | + | - | - | + |
| B1/74 | Tomentosa cherry | - | - | - | + | + | - | + | + | - | + | + |
| RR5 | Red raspberry | - | + | - | + | + | - | + | + | + | + | + |
| H7/73 | Crab apple | - | - | - | + | - | - | + | + | + | + | + |
| A25/75 | Mazzard cherry | - | - | - | - | + | - | - | - | - | - | - |
| M2/73 | Birch | + | + | - | + | + | - | + | + | + | + | + |
| B3/73 | Norway maple | - | + | - | + | - | - | + | + | + | + | + |
| N2/73 | Raspberry | - | + | + | + | + | + | + | + | + | + | + |
| S7/73 | Lippia | - | - | - | - | - | - | + | + | - | - | - |
| C5/73 | Mountain ash | - | - | - | + | + | - | + | + | + | + | + |
| E8/73 | Dahlia | - | + | - | + | - | + | + | - | + | + | - |
| CGlC | Pear | - | - | - | + | - | + | + | + | - | + | + |
| IIBV7 | Mums | - | - | - | + | + | + | + | + | - | + | + |
| T37 | Walnut | - | - | - | + | - | - | - | + | - | - | - |

Table 3. Tumor-inducing ability of eight Agrobacterium isolates, isolated from Lippia canescum galls, on 12 host species.

| Common names of host species tested | Isolates[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AB1/73 | AB2/73 | AB11/73 | AB12/73 | S1/73 | S2/73 | S7/73 | S9/73 |
| Apple | - | - | + | - | - | - | - | - |
| Bean | - | - | + | - | - | - | - | - |
| Beet | - | - | - | - | + | - | - | - |
| Bryophyllum | - | - | - | - | - | - | - | - |
| Plum | - | - | + | - | - | - | - | - |
| Daisy | - | - | - | - | - | - | - | - |
| Datura | + | - | + | + | + | + | + | - |
| Pea | - | - | - | - | - | - | + | - |
| Radish | - | - | + | - | - | - | - | - |
| Sunflower | + | - | - | - | + | - | - | - |
| Tomato | - | - | - | - | - | - | - | - |
| Lippia | - | + | - | + | + | + | + | - |

[a]Isolates AB1/73, AB2/73, AB11/73 and AB12/73 were all isolated from a single tumor. Similarly, isolates S1/73, S2/73 and S9/73 were all isolated from the same tumor. Isolate S7/73 was isolated from a third tumor.

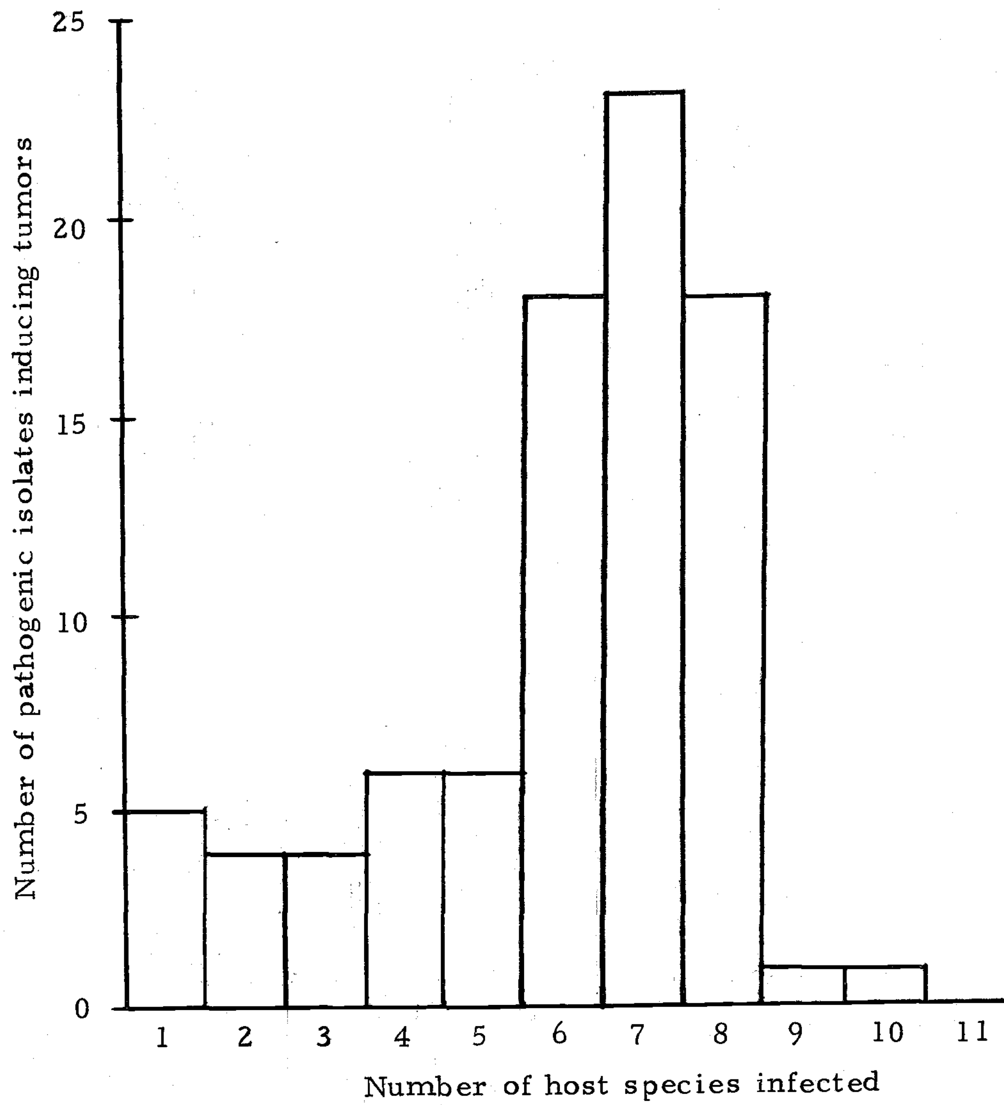


Figure 1. Frequency of tumor induction in 12 host species inoculated individually with 86 pathogenic Agrobacterium isolates.

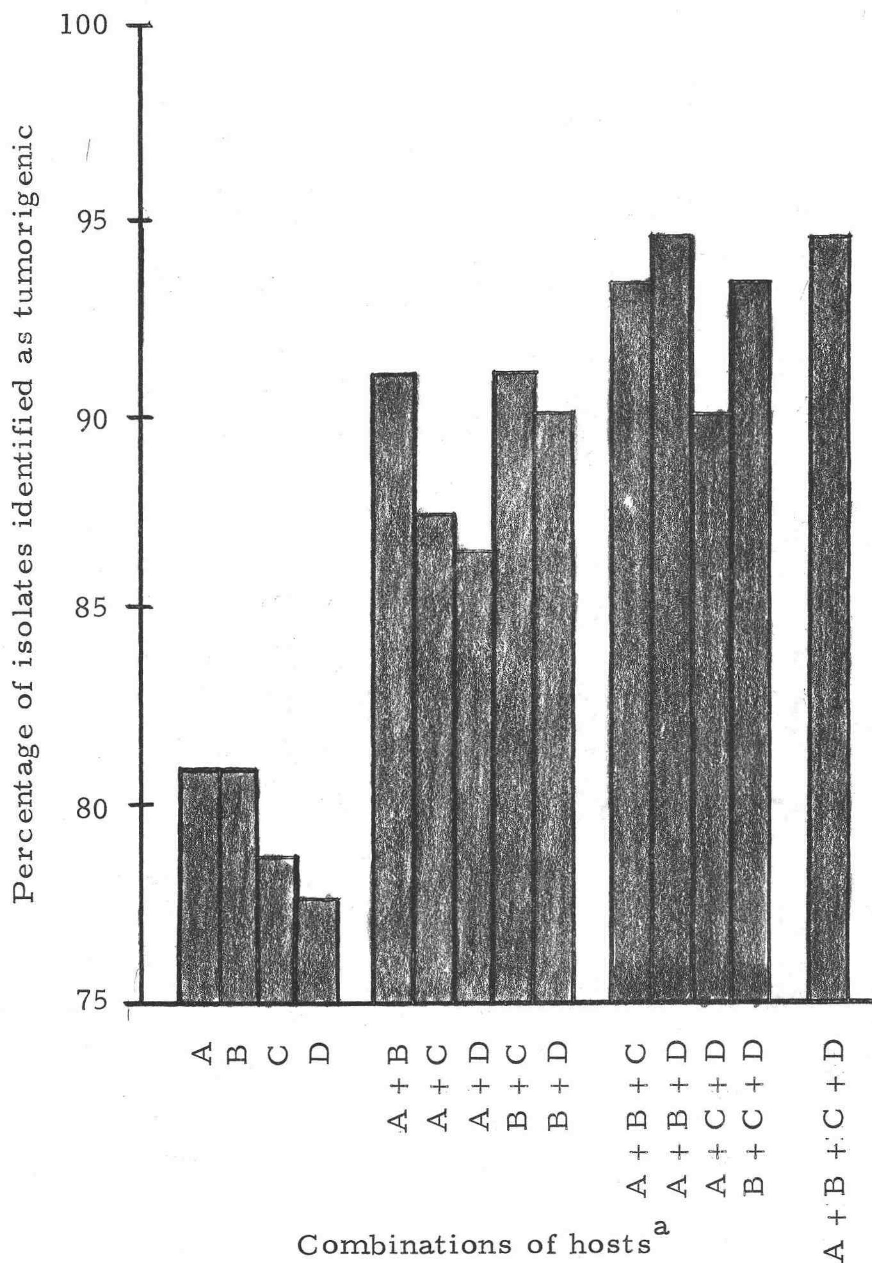


Figure 2. Use of selected hosts, either singularly or in combination, for identifying tumor induction of agrobacteria.

[a]A = tomato
B = datura
C = sunflower
D = bryophyllum

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# III. LACK OF CORRELATIONS BETWEEN PATHOGENICITY, NOPALINE AND OCTOPINE UTILIZATION AND AGROCIN-84 SENSITIVITY IN THE GENUS *AGROBACTERIUM*

## INTRODUCTION

A rapid and reliable method for identifying pathogenic agrobacteria has long been sought (3, 5, 9, 16, 24, 28). At present, inoculation of a living host is the only means of identifying pathogenicity. The reliability of this method recently has been questioned because of host specificity of *Agrobacterium* isolates (2). In addition, assay for pathogenicity on plants is tedious and time consuming. There remains a great need to develop a new method for rapid identification of phytopathogenic agrobacteria.

Recent evidence confirms that the genes for pathogenicity in *A. tumefaciens* are located on a very large plasmid called the tumor-inducing (Ti) plasmid (4, 23, 26, 27). Utilization (i.e., uptake and/or incorporation) of two unusual amino acids, nopaline [$N^2$-(1, 3-dicarboxypropyl)-L-arginine] (8) and octopine [$N^2$-(D-1-carboxyethyl)-L-arginine] (22), has been reported to be correlated with pathogenicity (14, 15, 19, 20) and the genes coding for utilization are on the Ti plasmid (23). Agrocin-84 sensitivity also has been reported as Ti borne (6, 26) and similarly correlated with pathogenicity (15, 18, 19, 20, 24). If a high degree of correlation between pathogenicity and

nopaline/octopine utilization or agrocin-84 sensitivity could be confirmed for agrobacteria, then a rapid method for identifying pathogenic *Agrobacterium* isolates could be developed. Therefore, a large number of *Agrobacterium* isolates of United States and world-wide origin (1) were examined for their ability to utilize nopaline and octopine and their sensitivity to agrocin-84.

## MATERIALS AND METHODS

### Organisms

All 178 *Agrobacterium* isolates tested were characterized previously and found to be representative of naturally-occurring United States (U.S.) and world-wide agrobacteria (1, 2). Approximately 40 of these isolates also were previously characterized by other laboratories for nopaline and octopine utilization and agrocin-84 sensitivity and were included as comparative controls. Working cultures of all isolates were kept on PDA (Difco) slants supplemented with 5.0 g $CaCO_3$/liter.

### Measurement of Utilization of Labeled Nopaline and Octopine

The method of Montoya et al. (23) was modified as follows. Inoculum was suspended in sterile distilled water to a final concentration of approximately $10^6$-$10^7$ colony forming units/ml. A 0.1 ml

aliquot was added to 0.4 ml of mannitol-glutamate medium supplemented with $^{3}$H-nopaline or $^{3}$H-octopine. All samples were incubated without shaking for 5 hours at 29 $\pm$ 1 C.

All samples were run twice. Nopaline and octopine negative isolates were re-examined for slow utilization of either amino acid analog by incubating the samples for a 24 hour period.

Measurement of Sensitivity to Agrocin-84

The method of Staver and Moore (unpublished) was used. A mannitol-glutamate medium (MG) base layer was overlaid with a MG medium (0.8% agar) containing at least $10^{8}$ colony forming units/plate. Three sterile antibiotic discs moistened with 50 microliters of a crude bacteriocin concentrate were added to each plate. The bacteriocin concentrate was prepared by rotary evaporation of a 100 ml supernatant of MG broth from a stationary culture of *A. radiobacter* 84 to 10 ml.

Calculation of Observed Correlations

Permutations of the five following characters were grouped as listed in Table 1: pathogenicity; 3-ketolactose reaction, the ability of an isolate to oxidize disaccharides to 3-ketoglycosides; utilization of nopaline and octopine; and sensitivity to agrocin-84. Three-ketolactose reaction was included as a character because of its

importance in identifying natural strain groupings among agrobacteria as described by Anderson and Moore (1). Standard correlations were calculated for all possible combinations of the above five characters.

## RESULTS

Correlation between pathogenicity and nopaline and/or octopine utilization was 0.35; between pathogenicity and agrocin-84 sensitivity, 0.31. Although 88% of the pathogenic agrobacteria utilized either or both guanido amino acids, 56% of the non-pathogens reacted similarly and consequently were responsible for the low correlation observed.

Isolates belonging to 25 of the 32 possible permutation groups have been identified (Table 1). Previously, only 11 groups had been reported (20). Although isolate reaction patterns for all five characters were very diverse, over 50% of the isolates fell into Groups 9, 10, 24 and 30. Over 50% of the Australian isolates described by Kerr and Roberts (20) also fell into the same four groups. Isolates of similar geographic origin or that were isolated from the same plant species were randomly distributed among all groups.

All eight _A. rhizogenes_ isolates which produced hairy root (HR) via the carrot assay (11) fell into Group 14. The other 11 named _A. rhizogenes_ isolates which failed to induce HR were randomly

dispersed among the remaining 24 groups identified. All confirmed *A. rhizogenes* isolates were octopine utilizers as previously reported (14).

## DISCUSSION

The reported high correlations between pathogenicity and nopaline and/or octopine utilization and between pathogenicity and agrocin-84 sensitivity were not confirmed by our data. The differences between these reported correlations are due to both the number and the kinds of agrobacteria examined. The majority of previously examined isolates were pathogens. Since 88% of the 90 pathogens characterized in this study utilized either nopaline or octopine or both, and the genes coding for utilization of both guanido amino acids are coded for on the tumor-inducing (Ti) plasmid, the reported correlations would naturally be expected. However, 56% of the 85 non-pathogenic isolates also utilize either or both guanido amino acids. Therefore, when a sufficiently large sample of both pathogenic and non-pathogenic agrobacteria was examined, the natural correlations become evident.

Although genes coding for pathogenicity, nopaline and octopine utilization and agrocin-84 sensitivity have been shown to be located on the Ti plasmid, the lack of correlations observed in this study between these characters may be due to one or more of several

possible reasons. Plasmid mobility, as observed between *Agrobacterium* isolates *ex planta* (7, 10, 20, 21, 23) and *in planta* (17, 23, 26), can result in transfer of one or more characters to randomly diverse agrobacteria. Currier and Nester (4) reported that *Agrobacterium* isolates with 10% chromosomal homology can have greater than 80% plasmid homology. The converse was also reported. Incomplete plasmid transfer, gene mutation or deletion, incorporation of plasmid genes into the chromosome or factors affecting plasmid-gene expression could similarly contribute to the lack of observed correlations.

The functions which octopine and nopaline play in the tumorigenic process are unknown, even though tumor growth enhancement by both guanido amino acids has been reported (12, 13, 25). These unusual amino acids also may be involved in derepression of genes responsible for plasmid transfer between agrobacteria (7, 21). Regardless of their possible functions however, our data show that neither substance is universally required for pathogenicity among agrobacteria.

A paper by Kerr and Roberts (20) has appeared since the work reported here was completed. Seventy isolates, principally of Australian origin, were tested for utilization of nopaline and octopine and sensitivity to agrocin-84. Although exceptions to the previously reported correlations were also reported, only 11 of the possible 32

permutation groups were identified. Furthermore, Kerr and Roberts reported that none of their 3-ketolactose positive [K(+)] isolates utilized both nopaline and octopine and suggested that K(+) isolates could carry a "nopaline plasmid" or an "octopine plasmid," but not both. This assumption is incorrect since two K(+) isolates were identified which are capable of utilizing both nopaline and octopine. Thirty-eight K(-) isolates were found also to utilize both guanido amino acids. We found no apparent differences in isolate reactions to the five characters examined between the two strains of agrobacteria, as identified by the 3-ketolactose reaction (1).

Kerr (19) has proposed a genetic model to explain pathogenicity in Agrobacterium and tumor induction in plants. Two of the four assumptions made in the model are: i) close correlation exists between pathogenicity and either octopine or nopaline metabolism, and ii) sensitivity to bacteriocin-84 is closely correlated with nopaline metabolism. The correlation values calculated in this study for assumptions (i) and (ii) are 0.35 and 0.23, respectively, suggesting that the proposed model is incomplete for explaining pathogenicity in Agrobacterium. Additional information, regarding frequency of plasmid gene transfer, identification of other plasmid genes and the functions of plasmid gene products, must be better understood before a model for pathogenicity and tumor induction can be proposed.

# SUMMARY

One hundred seventy-eight previously characterized *Agrobacterium* isolates were tested for nopaline and/or octopine utilization and agrocin-84 sensitivity. Low correlations were observed between all combinations of these three characters, the 3-ketolactose test and pathogenicity. Although 88% of the pathogenic agrobacteria utilized either or both guanido amino acids, 56% of the non-pathogens reacted similarly, resulting in a correlation coefficient of 0.35 for pathogenicity and nopaline/octopine utilization. A correlation of 0.31 was observed for pathogenicity and agrocin-84 sensitivity.

Of 32 possible permutation groups of the five characters examined, isolates belonging to 25 groups were identified. Isolates were randomly distributed among all 25 groups regardless of geographic origin or plant species of isolation. All *A. rhizogenes* isolates, forming hairy root via the carrot assay, fell into Group 14 and utilized octopine. Possible reasons for the lack of high correlations between the plasmid-coded characters are discussed.

Table 1. Reaction patterns of 175 agrobacteria to five tests assayed.

| Group no. | Number of isolates with each reaction pattern | Possible reaction patterns to the 5 tests | | | | |
|---|---|---|---|---|---|---|
| | | Pathogenicity[a] | 3-Ketolactose reaction[a] | Nopaline utilization | Octopine utilization | Agrocin 84 sensitivity |
| 1 | 0 | + | + | + | + | + |
| 2 | 0 | + | + | + | + | - |
| 3 | 9 | + | + | + | - | + |
| 4 | 4 | + | + | + | - | - |
| 5 | 3 | + | + | - | + | + |
| 6 | 9 | + | + | - | + | - |
| 7 | 5 | + | + | - | - | + |
| 8 | 2 | + | + | - | - | - |
| 9 | 14 | + | - | + | + | + |
| 10 | 21 | + | - | + | + | - |
| 11 | 1 | + | - | + | - | + |
| 12 | 5 | + | - | + | - | - |
| 13 | 2 | + | - | - | + | + |
| 14 | 11 | + | - | - | + | - |
| 15 | 1 | + | - | - | - | + |
| 16 | 3 | + | - | - | - | - |
| 17 | 1 | - | + | + | + | + |
| 18 | 1 | - | + | + | + | - |
| 19 | 2 | - | + | + | - | + |
| 20 | 10 | - | + | + | - | - |
| 21 | 0 | - | + | - | + | + |
| 22 | 2 | - | + | - | + | - |
| 23 | 4 | - | + | - | - | + |
| 24 | 28 | - | + | - | - | - |
| 25 | 0 | - | - | + | + | + |
| 26 | 3 | - | - | + | + | - |
| 27 | 0 | - | - | + | - | + |
| 28 | 0 | - | - | + | - | - |
| 29 | 3 | - | - | - | + | + |
| 30 | 26 | - | - | - | + | - |
| 31 | 0 | - | - | - | - | + |
| 32 | 5 | - | - | - | - | - |

[a]Each isolate's ability to form 3-ketoglycosides and its pathological characters has been previously reported (1, 2).

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