Comparison of Thirty-Seven Strains of Vd-3 Bacteria with *Agrobacterium radiobacter*: Morphological and Physiological Observations

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Thirty-seven cultures of Vd-3 bacteria, isolated from clinical specimens, were characterized morphologically and physiologically. The cultures produced positive reactions when tested for oxidase, urease, nitrate reduction, phenylalanine deaminase, oxidative metabolism of carbohydrate substrates, and 3-ketolactose production. These peritrichously flagellated microorganisms were isolated primarily from the respiratory tract. When compared to authentic strains of *Agrobacterium*, they appeared to be most similar to *A. radiobacter*. Gas-liquid chromatography of trimethylsilyl derivatives of whole-cell hydrolysates of some of the Vd-3 strains and *A. radiobacter* yielded nearly identical elution patterns. The Vd-3 cultures were identified as probable strains of *A. radiobacter*. A method is presented for differentiating cultures of *A. radiobacter* from other similar bacteria encountered in clinical specimens. Although these bacteria rarely occur in clinical specimens, the clinical microbiologist should be familiar with their outstanding characteristics.

Microorganisms of the genus *Agrobacterium* are of primary importance because of their significance in agriculture. Their ability to produce crown-gall tumors is well documented. In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (3), four species are listed in the genus *Agrobacterium*. Three of these microorganisms, *A. tumefaciens*, *A. rhizogenes*, and *A. rubi*, are designated as phytopathogens. Only *A. radiobacter* has not been observed to demonstrate plant pathogenicity. *Agrobacterium* species are usually associated with soil and, thus, they would not be expected to be isolated from clinical specimens.

Lautrop (10) characterized and identified 10 bacterial cultures, which he had isolated from clinical specimens, as *Agrobacterium* species. However, only one of the cultures produced the typical crown-gall lesions; he identified it as *A. radiobacter* var. *tumefaciens*. He concluded that no evidence indicated that these bacteria either initiated or participated in the patients' diseases.

During the past 12 years, the Special Bacteriology Section (SBS) at the Center for Disease Control has received 37 cultures with characteristics similar to those described for *Agrobacterium* species. These bacterial cultures, arbitrarily designated Vd-3, and the type strain of *A. radiobacter* ATCC 19358 were investigated morphologically and biochemically to determine the extent of their relatedness.

MATERIALS AND METHODS

Bacteria. The bacterial cultures were obtained from the stock culture collection maintained by the SBS. They were identified from clinical isolates submitted to this laboratory for further investigation (Table 1). The type culture of *A. radiobacter* ATCC 19358 was obtained from the American Type Culture Collection.

Media and procedures. The biochemical tests were those routinely used in the SBS for the study of microorganisms submitted for identification. The media preparation and the procedures used in investigating the biochemical characteristics have been previously described (4, 9, 12). The production of 3-ketolactose was detected by the method of Bernaerts and DeLey (1). The oxidation of potassium gluconate was determined by the procedure of Moore and Pickett (11). All agar slant culture media were inoculated with one drop of an 18- to 24-h heart infusion broth culture (Difco).

Gas-liquid chromatography. The freeze-dried cells were hydrolyzed by the technique of Farshy and Moss (5). The dried hydrolysate was dissolved in 0.5 ml of TRI-SIL (Pierce Chemical Co.). The vial (Reacti-Vial, Pierce Chemical Co.) containing the sample was flushed with nitrogen, capped, and placed in a 65°C water bath for 15 min. After the sample was incubated, it was reduced to dryness under nitrogen at 50°C. The dried residue was dissolved in 1 ml of hexane, and the final volume was reduced to 0.1 ml under nitrogen.

The samples were analyzed with a Hewlett-Packard 402-B gas chromatograph equipped with dual-flame ionization detectors and 2.4-m glass columns with 3-mm inner diameters. The detector tempera-

TABLE 1. Sources of Vd-3 bacterial isolates

SBS no.	Source	Origin
B6016	Sputum	Ga.
C3703	Sputum	St. Louis, Mo.
C5763	Sputum	Wis.
C8178	Sputum	Neb.
D1314	Sputum	Colo.
D3907	Sputum	Mo.
D2927	Sputum	R.I.
C5765	Sputum	Wis.
D847	Leg abscess	Mo.
B9542	Wound (unspecified)	La.
C9686	Hand cut	Pa.
C7290	Hand wound	Calif.
C7258	Lesion (unspecified)	Tex.
C5382	Blood	Tex.
D1875	Blood	Mich.
D4205	Blood	Conn.
C7291	Bronchial washings	Hawaii
D3125	Bronchial washings	Idaho
B3719	Tracheal aspirate	Ga.
C1430	Eye	Belgium
C1429	Eye	Belgium
8914	Eye	La.
C3125	Urine	Me.
D917	Urine	Mo.
D2922	Urine	Calif.
C4899	Synovial fluid	Tenn.
8729	Ear	Pa.
A6597	Vagina	S.C.
B3731	Colon	Colo.
C1266	Contact lens solution	Calif.
D965	Rat spleen	South Africa
D1734	Endometrium	Md.
D2023	Antrum (unspecified)	Conn.
D2565	Sewage	CDC ^a
D4024	Right tube & ovary	Hawaii
D3253	Catheter, i.v.	Ariz.
D6578	Urethra	Calif.

^a CDC, Center for Disease Control.

ture was 260°C. Helium, with a flow rate of 40 ml/min, was used as the carrier gas. Sample analysis was done on columns consisting of 3% OV-1 on Gas Chrom Q, 100- to 120-mesh (Applied Science Labs, Inc.). The column temperature was held for 4 min at 130°C and then programmed to 250°C at the rate of 3°C/min. The peak areas and the percentages of each eluted component in the samples were determined with a Hewlett-Packard 3380 A reporting integrator.

RESULTS

After 24 h of incubation at 35°C on 5% rabbit blood agar plates, the colonies of the *A. radiobacter* and Vd-3 cultures were punctate, circular, and convex, and had an entire edge. The cellular growth was glossy, butyrous, and nearly opaque. Lysis was noted on approximately 20% of the plates.

All of the strains were gram-negative rods (ca. 0.4 to 0.8 by 1.3 to 2.5 μ m), with parallel sides and rounded ends. A few slightly curved

cells were also observed. They were motile by peritrichous flagellation (Fig. 1). If only one flagellum was observed, it was usually laterally located. The cells primarily occurred as single cells.

Some of the physiological characteristics determined for each of these bacterial cultures are listed in Table 2. All of the strains were 3-ketolactose positive. Both the *A. radiobacter* and Vd-3 cultures were oxidase positive and catalase positive, and both hydrolyzed urea and esculin, alkalized citrate, and produced phenylalanine deaminase. They grew on MacConkey agar, but not on cetrinide agar. Nitrate was reduced by each of the cultures. *A. radiobacter* and only 60% of the Vd-3 cultures, however, reduced nitrite, with no detectable gaseous products; gas production during denitrification was not observed when the semisolid medium of Stanier et al. (14) was used. After

TABLE 2. Some characteristics of *Agrobacterium radiobacter* and Vd-3 cultures

Test or substrate ^a	Test results ^b	
	<i>A. radiobacter</i>	Vd-3
Citrate alkalization	+	+
3-Ketolactose production	+	+
2-Ketogluconate production	—	—
Urea hydrolysis	+	+
Phenylalanine deaminase	+	+
Catalase	+	+
Oxidase	+	+
Gelatin liquefaction	—	—(1) ^b
Growth on:		
MacConkey agar	+	+
SS agar	—	—(10)
Cetrinide agar	—	—
6% NaCl broth	—	—(1)
TGY agar at 25°C	+	+
TGY agar at 35°C	+	+
TGY agar at 42°C	—	—(12)
Nitrate reduction	+	+
Nitrite reduction	+	+(14)
Indole production	—	—
Esculin hydrolysis	+	+
Lysine decarboxylase	—	—
Arginine dihydrolase	—	—(4)
Ornithine decarboxylase	—	—
Acid production from:		
Glucose OF, open tube	+	+
Glucose OF, closed tube	—	—
Litmus milk, clearing zone at surface	+	+

^a Abbreviations: OF, oxidative-fermentative; SS, salmonella-shigella; TGY, tryptone-glucose-yeast extract.

^b The number inside the parentheses indicates the number of strains that deviated from the recorded result.

FIG. 1. Illustration of the flagellar arrangement of the Vd-3 bacteria (culture C1430; $\times 1,700$).

the cultures were inoculated into litmus milk, all of them produced a serum-like zone in the top 0.25 to 0.5 cm of the liquid. After each culture had been inoculated into duplicate tubes of glucose oxidative-fermentative medium, one of the tubes was sealed with 1 to 2 cm of petrolatum. Acid production was detected in each of the unsealed tubes, but was not observed in any of the sealed tubes.

The oxidation of various carbohydrates with the concomitant production of acid was observed (Table 3). Each strain produced acid from most of the carbohydrates tested. *A. radiobacter* did not produce acid from dulcitol, inulin, dextrin, glycogen, erythritol, starch, or cellulose. Significant numbers of the Vd-3 bacteria, however, produced acid in dulcitol (18%) and dextrin (29%). Moreover, melezitose was not oxidized by 35% of the Vd-3 cultures.

Observation of the gas-liquid chromatograms of the trimethylsilyl profiles revealed slight variation between the *A. radiobacter* and Vd-3

strains examined (Table 4). The variations were exhibited in quantitative rather than qualitative peak profiles. The trimethylsilyl derivatives did not vary considerably either in the number of peaks or in the relative peak retention times. Twenty-eight major peaks ($\geq 1\%$ of the total peak area) and a total of 33 to 35 peaks were detected in the profiles of the microorganisms. Each of the cultures examined had 13 common peaks, eluting at 18.2, 23.5, 24.5, 26.8, 27.4, 29.1, 29.6, 32.9, 35.6, 38.3, 40.8, 43.3, and 45.8 min, which accounted for 71 to 78% of the total peak area of the samples. The peaks detected at 18.2 and 23.5 min represent 30 to 40% of the total peaks in each of the cultures. The elution time for each of the peaks recorded did not vary from strain to strain by more than 0.1 min.

DISCUSSION

The results obtained in this study indicate that the Vd-3 bacterial cultures are very simi-

TABLE 3. Carbohydrate oxidation patterns of *Agrobacterium radiobacter* and Vd-3 cultures

Substrate ^a	Acid production	
	<i>A. radiobacter</i>	Vd-3
Glucose	+	+
Xylose	+	+
Mannitol	+	+
Lactose	+	+
Sucrose	+	+
Maltose	+	+
Glycerol	+	+
Salicin	+	+
L-Arabinose	+	+
Adonitol	+	+
Dulcitol	—	— (6) ^b
Galactose	+	+
Fructose	+	+
Mannose	+	+
Rhamnose	+	+
Trehalose	+	+
Raffinose	+	+
Sorbitol	+	+
Inositol	+	+
Cellobiose	+	+
Inulin	—	— (1)
Dextrin	—	— (10)
Glycogen	—	—
Erythritol	—	—
Melibiose	+	+
Melezitose	+	— (12)
Starch	—	— (1)
Cellulose	—	—

^a The filter-sterilized carbohydrates were added aseptically to the oxidative-fermentative medium to a concentration of 1%. Incubation was for 7 days at 35°C.

^b The number inside the parentheses indicates the strains which deviated from the recorded result.

lar to *A. radiobacter* ATCC 19358. The biochemical and morphological characteristics of all of the cultures used in this study are nearly identical. Moreover, chromatography of trimethylsilyl derivatives of whole-cell hydrolysates yielded nearly identical patterns. Intraspecies similarities in derivative profiles have been shown to be characteristic of some bacterial species (2, 5, 6, 13).

Therefore, 16 of the Vd-3 cultures were submitted to K. Kersters for an expert opinion. After examining these bacteria, Kersters (personal communication) concluded that these microorganisms belonged in his *Agrobacterium* taxonomic cluster 1 (8), which is composed of 3-ketolactose-positive organisms that may be either tumor-inducing or nonphytopathogenic strains. After inoculating plants, he determined that the Vd-3 cultures, which were non-tumor-inducing, were strains of *A. radiobacter* var. *radiobacter*.

Lautrop (10) indicated that only 4 of the 10 clinical isolates that he studied were 3-ketolactose producers. We also observed several 3-ketolactose-negative cultures which were otherwise identical to the Vd-3 bacteria. Kersters examined 15 of these organisms. Kersters (personal communication) found that they did not appear to be similar to any *Agrobacterium* strains that he had observed.

Cultures of *A. radiobacter* may be confused with several microorganisms that are encountered in clinical specimens. In particular, several saccharolytic, nitrate-positive species of

TABLE 4. Relative percentage of peaks obtained from chromatograms of trimethylsilyl derivatives prepared from whole-cell hydrolysates^a

Retention time (min)	Relative percentage ^a					
	<i>A. radiobacter</i> ATCC 19358	Vd-3				
		B9542	8914	A6597	C3125	C1430
8.3	T	T	0.5		T	T
10.3	0.9	0.6	1.3	1.5	2.1	0.5
11.5	0.8	T	T	0.6	3.2	1.9
13.0	T	T	0.6	T	T	T
14.6	T	T	T	T	T	0.6
17.1	1.6	0.7	1.2	0.8	1.1	0.6
18.2	17.1	9.0	15.5	13.1	18.2	13.9
19.5	4.2	3.7	1.7	2.3	1.1	0.9
20.3	2.9	2.3	1.9	1.5	0.6	0.9
20.7	T	T	T	T	T	0.8
21.2	0.8	0.9	0.9	2.3	5.1	0.6
22.1	0.8	0.6	T		T	0.7
23.5	23.4	21.1	21.5	18.5	20.9	16.2
24.5	4.4	6.2	5.1	2.6	2.1	1.7
25.2	1.1	0.9	1.2	1.0	0.9	1.0
26.0	2.3	1.5	1.4	0.8	1.5	0.6
26.8	7.8	7.0	3.6	4.2	3.9	1.8
27.4	1.9	2.2	2.4	2.9	1.7	3.1
29.1	2.6	4.6	6.0	3.2	3.6	5.7
29.6	4.4	3.2	4.3	1.5	2.3	1.6
30.0	1.4	2.3	2.3	3.9	3.5	4.1
30.6		0.8	0.8	T	T	
31.2	1.1	1.4	1.1	0.9	0.9	0.5
31.8	0.9	1.0	0.9	1.3	1.6	T
32.9	3.4	5.7	3.2	5.1	3.6	5.6
35.6	1.6	3.6	3.7	4.2	5.2	4.1
38.3	2.3	4.4	4.4	5.8	2.7	5.0
40.8	1.6	3.1	3.8	2.6	4.9	3.8
42.4	T	3.4	T	0.9	T	1.9
43.3	1.3	2.8	2.9	3.5	2.3	5.8
44.3	0.5				T	0.9
44.9	0.5	0.6	0.5			0.9
45.8	1.1	2.2	1.9	3.7	1.2	4.8
47.6	1.5	0.5	0.8	1.2	0.7	1.4
48.8	2.2	1.4	1.0	2.8	0.9	3.1
52.6	1.1	0.7	0.8	0.5	0.6	1.7

^a Relative percentage reflects the amount of each component in the sample. T, Relative percentage less than 0.5%.

TABLE 5. Comparison of *Agrobacterium radiobacter* to other similar oxidative microorganisms which are isolated from clinical specimens^a

Test	<i>A. radiobacter</i> (38)	Group Vd (91)	Group Va-1 (70)	<i>P. pickettii</i> (54)	<i>P. cepacia</i> (55)	<i>P. pseudomallei</i> (70)	<i>P. stutzeri</i> (97)	<i>A. xylosoxidans</i> (53)	<i>P. fluorescens</i> (155)
Acid production from:									
Glucose OF ^b	+(100)	+(93)	+(100)	+(100)	+(100)	+(100)	+(98)	-(44)	+(100)
Xylose OF	+(100)	+(100)	+(100)	+(100)	+(98)	+(97)	+(99)	+(98)	+(100)
Mannitol OF	+(100)	+(51)	-(0)	-(0)	+(62)	+(100)	+(53)	-(0)	+(55)
Lactose OF	+(100)	-(0)	-(0)	-(0)	+(98)	+(100)	-(0)	-(0)	-(28)
Sucrose OF	+(100)	-(29)	-(0)	-(0)	+(64)	+(64)	-(0)	-(0)	-(48)
Maltose OF	+(100)	-(34)	+(100)	-(0)	+(100)	+(100)	+(70)	-(0)	-(2)
Flagellar arrangement ^c	P	P	1	1	2	2	1	P	2
3-Ketolactose production	+(100)	-(0)	-(0)	-(0)	-(0)	-(0)	-(0)	-(0)	-(0)

^a The number inside the parentheses beneath the culture name refers to the number of cultures tested. The number beside the recorded result for the test refers to the percentage of positive results obtained.

^b OF, Oxidative-fermentative medium.

^c The symbols for flagellar arrangement indicate: 1, 1 to 2 polar flagella; 2, more than 2 polar flagella; and P, peritrichous flagellation.

Pseudomonas and *Achromobacter*-like cultures appear similar to *A. radiobacter*. A comparison of selected characteristics, however, provides a means of differentiation (Table 5). If the 3-ketolactose test is not performed, the oxidation of the carbohydrates, mannitol, lactose, and maltose, will separate *A. radiobacter* from each of these bacteria except *Pseudomonas cepacia* and *Pseudomonas pseudomallei*. These latter two organisms can be eliminated by flagellar staining. Moreover, Hugh and Gilardi (7) indicated that *P. cepacia* cultures decarboxylate lysine (93% positive). *A. radiobacter* is lysine decarboxylase negative. The recommended test for *A. radiobacter* identification is that for 3-ketolactose production.

Each of the bacterial cultures used in this study, except the type strain of *A. radiobacter*, was isolated from clinical specimens. We have been unable, however, to secure any information that might imply that these cultures can initiate a human infection. Lautrop (10) also surmised that none of the bacterial isolates that he studied were of clinical significance. He concluded that these bacteria occur either as incidental inhabitants in the patient or as contaminants introduced during sample manipulation. The case histories we have accumulated tend to confirm Lautrop's conclusion.

The probable frequency with which these bacteria are encountered in the clinical laboratory is difficult to determine. We cannot make a reliable estimate because the cultures submitted to the SBS are not representative of the percentages of the cultures encountered in the clinical laboratory. Laboratories that submit cultures to the SBS send only those cultures

which are unusual or which present identification problems. The receipt of only 37 cultures of agrobacteria over 12 years, however, indicates that they are infrequently encountered. Nevertheless, the clinical microbiology laboratorian should become familiar with the outstanding characteristics of these microorganisms.

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LITERATURE CITED

- Bernaerts, M. J., and J. DeLey. 1963. A biochemical test for crown-gall bacteria. *Nature* (London) 197:406-407.
- Bovre, K., R. Hytta, E. Jantzen, and L. O. Froholm. 1972. Gas chromatography of bacterial whole cell methanolysates. III. Group relations of *Neisseria* and *Moraxellae*. *Acta Pathol. Microbiol. Scand. Sect. B* 80:683-689.
- Buchanan, R. E., and N. E. Gibbons (ed.). 1974. *Bergey's manual of determinative bacteriology*, 8th ed. Williams and Wilkins, Baltimore, Md.
- Ewing, W. H., and B. R. Davis. 1970. Media and tests for differentiation of *Enterobacteriaceae*. Center for Disease Control, Atlanta, Ga.
- Farshy, D. C., and C. W. Moss. 1970. Characterization of clostridia by gas chromatography: differentiation of species by trimethylsilyl derivatives of whole-cell hydrolysates. *Appl. Microbiol.* 20:78-84.
- Froholm, L. O., E. Jantzen, R. Hytta, and K. Bovre. 1972. Gas chromatography of bacterial whole cell methanolysates. II. A taxonomic evaluation of the method for species of *Moraxella*. *Acta Pathol. Microbiol. Scand. Sect. B* 80:672-682.
- Hugh, R., and G. L. Gilardi. 1974. *Pseudomonas*, p. 250-269. In E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), *Manual of clinical microbiology*, 2nd ed. American Society for Microbiology, Washington, D.C.

8. Kersters, K., J. DeLey, P. H. A. Sneath, and M. Sackin. 1973. Numerical taxonomic analysis of *Agrobacterium*. J. Gen. Microbiol. 78:227-239.
9. King, E. O. 1967. The identification of unusual gram-negative bacteria (revised by R. E. Weaver). Center for Disease Control, Atlanta, Ga.
10. Lautrop, H. 1967. *Agrobacterium* spp. isolated from clinical specimens. Acta Pathol. Microbiol. Scand. 187(Suppl.):63-64.
11. Moore, H. B., and M. J. Pickett. 1960. The *Pseudomonas-Achromobacter* group. Can. J. Microbiol. 6:35-42.
12. Riley, P. S., H. W. Tatum, and R. E. Weaver. 1972. *Pseudomonas putrefaciens* isolates from clinical specimens. Appl. Microbiol. 24:798-800.
13. Riley, P. S., and R. E. Weaver. 1975. Recognition of *Pseudomonas pickettii* in the clinical laboratory: biochemical characterization of 62 strains. J. Clin. Microbiol. 1:61-64.
14. Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43:159-271.