Relationships Between *Xanthomonas c.* pv. *manihotis*, *X. c.* pv. *cassavae* and Colombian Yellowish Isolates

As Reflected in Physiological, Biochemical, and Serological Tests.

F. N. Elango, Centro Internacional de la Papa, Lima, Peru
J. C. Lozano, CIAT, Cali, Colombia
J. F. Peterson, McGill University, Quebec, Canada

Abstract

Physiological/biochemical tests were inadequate in separating *Xanthomonas campestris* pv. *manihotis*, *X. c.* pv. *cassavae*, and some Colombian yellowish isolates. However, serological techniques such as the Ouchterlony double diffusion and direct immunofluorescence indicated antigenic differences between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae*. The Colombian yellow isolates were serologically similar to *X. c.* pv. *cassavae* but not to *X. c. manihotis*. Therefore, retaining the distinction between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* is probably justified.

Introduction

Xanthomonas campestris pv. *manihotis* (Berthet and Bondar 1915) Dye 1978 and *X. c.* pv. *cassavae* (Wiehe and Dowson 1953) Maraite and Weyns 1979 are two of four bacterial pathogens which attack cassava (*Manihot esculenta* Crantz) (24,37). While *X. c.* pv. *manihotis* occurs in most cassava growing areas of the world (25), *X. c.* pv. *cassavae* is limited to the African highlands of Rwanda and Kenya at 1,600 m (26) and, possibly, sea-level in the eastern plains of Colombia (4). Because the early leaf spot symptoms are so similar, workers frequently have difficulty distinguishing the two pathogens. It has been reported that the pathogens are synonymous (29), but other evidence (16, 26) suggests the contrary.

Taxonomically, *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* are grouped in Bergey's Manual of Determinative Bacteriology (2) under *X. campestris* group, but as two distinct pathovars. However, the newly proposed nomenclature which came into use January 1, 1980 (39) considers *X. cassavae* as a *nomen dubium*, probably on the basis of controversial evidence (29). Therefore, the relationship between these two pathogens needs a clear distinction to avoid confusion for quarantine purposes (26). Moreover, reports that *X. c.* pv. *cassavae* may have evolved from *X. c.* pv.

manihotis need to be confirmed. "Deeper" taxonomic studies have been suggested to resolve this problem (26) and such studies could include genetics, bacteriophage, and serology (36).

This study was undertaken in a continued effort to clarify the relationship between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae.*

Materials and Methods

Physiological and biochemical tests were used, methods and references are indicated in Table 1. The replica plating technique (22) was used to study the utilization of carbohydrates, nitrates, fatty acids, amino acids, amines, organic acids, and sodium and tetrazolium chloride salts.

Antisera were produced against *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae.* Cultures used for antisera production were prepared according to the method of Allan and Kelman (1), i.e., using glutaraldehyde fixed whole cells to immunize New Zealand white rabbits. Part of the crude antiserum was preserved for the agglutination test (35) and the Ouchterlony double diffusion assay (19), while another portion was fractionated following the methods of Allan and Kelman (1) and Cherry (5). The precipitated globulin was conjugated with Fluorescein isothiocyanate (FITC) (5). Samples were stored in vials at -20°C until needed.

To determine the serological relationship between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* and some Colombian xanthomonads, isolates were grown for 48 hours on Pseudomonas isolation agar (7). Dilutions of samples were smeared onto alcohol cleaned slides, air-dried, heat fixed and later stained with fluorescent antibody conjugate. The stained preparations were examined under an X 100 objective fitted to an Orthomat microscope with an oil immersion condenser.

The relationship between *X. c.* pv. *manihotis, X. c.* pv. *cassavae,* and the Colombian yellowish isolates was studied by the method of Klement (19). All gel-diffusion tests were made at room temperature (25°C) Photographs of double diffusion plates were taken in an immunodiffusion Camera (Cordis) using Polaroid 107 black and white film.

Results

Results shown in Table 2 indicate that the three xanthomonads could not be separated adequately by physiological and biochemical tests. However, all tests marked with two asterisks showed some differential value between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* on the one hand and the Colombian isolates on the other hand. The differences cited were mainly in the rate of utilization of several carbohydrate and other nutritional sources. Other tests showing differences included the hypersensitivity tests, tolerance to tetrazolium chloride salt, growth rate, pigmentation, and sodium polypectate. Tests marked with one asterisk showed an inconsistent differential value. Fatty acids, amino acids, amines, and organic acids were not useful in separating the species.

Glutaraldehyde-fixed whole cells were used as antigens, and they induced the production of highly specific antisera. The agglutination titre of

Table 1. Tests and methods used for physiological and biochemical tests.

Test	Method (Reference)
Oxidades	(7, 21)
Catalase	(8, 35)
Mode of glucose utilization	(15)
Gelation liquefaction	(17, 28)
Hydrolysis of starch	(17)
Indole production	(17)
Nitrate reduction	(17)
Tyrosinase	(8, 38)
Hydrolysis of Lipase	(31)
NaCl tolerance	(30)
Tetrazolium chloride tolerance	(23)
Urease	(6, 13)
H_2S production	(17)
Carbohydrate utilization	
Nitrate utilization	
Fatty acids	(27)
Organic acids	
Amino acids	
Amines	
Soft rot of potato and cassava roots	(25)
Tobacco hypersensitivity reaction	(20)
Pectate liquefaction	(13)
Hydrolysis of casein	(12)
Litmus milk reaction	(7, 17)
Ammonia production	(17)
Phenylalanine deaminase	(32)
Methyl red and Voges proskeur	(17)
Arginine dihydrolase	(27)
Hydrolysis of Esculin	(34)
Sodium polypectate	(3)
B-glucosidase	(14)
Levan production	(27)
Fluorescence on King's B Medium	(18)
Growth rate	
Pigmentation	
Sensitivity to antibiotics	(7)

Table 2. Physiological/biochemical characterization of *Xanthomonas campestris manihotis*, *X. c.* pv. *cassavae*, and some Colombian yellowish isolates.

Test	Reaction		
	X. manihotis	*X. cassavae*	Colombian Yellowish isolates
Carbohydrate utilization[a]	+–[c]	+	++
Nitrate utilization	–	–	–
Amines	+	+	+
Amino acids	+	+	+
Organic acids	–	–	–
Antibiotic sensitivity[b]	+	+	++
Hydrolysis of casein	–	–	–
Hydrolysis of gelatin	+	+	+
Hydrolysis of starch	+	+	+
Litmus milk reaction	+	+	+
Production of ammonia	+	+	+
Production of H_2S	–	–	–
Production of indole	–	–	–
Nitrate reduction	–	–	–
Methyl red and V.P. test	–	–	–
Mode of glucose utilization	Oxidative	Oxidative	Oxidative
Lipase	+	+	+
Catalase	+	+	+
Oxidase	–	–	–
Arginine dihydrolase	–	–	–
Phenylalanine deaminase	+	+	+
Tyrosinase	–	–	–
Urease	–	–	–
Aesculin hydrolysis[b]	+	+	++
B-glucosidase	+	+	+
Soft rot of potatoes & cassava roots	–	–	–
Levan production	+	+	+
Hypersensitivity in tobacco leaves[a]	–	–	+
Salt tolerance (Max.)	2.5°/o	2.5°/o	2.5°/o
Tetrazolium chloride (tolerance [a]Max.)	0.02°/o	0.02°/o	0.1°/o
Sodium polypectate[a] utilization	–	–	+
Fluorescence on King's B. medium	–	–	–
Growth rate[a]	moderate	slow	fast
Yellow pigment[a]	–	+	+

[a] Tests with distinct differential value.

[b] Tests with non-consistent differential value.

[c] ++ = strongly positive; + = positive; – = negative.

Table 3. Serological identity between *Xanthomonas campestris* pv. *manihotis* (Berthet and Bondar 1915) Dye 1978, *X. c.* pv. *cassavae* Wiehe and Dowson 1953 (Maraite and Weyns 1953) and some Colombian yellowish isolates.

Antigen (Whole cells) 1×10^9/ml	Antibody Reaction[a,b]			
	Double Diffusion		Immunofluorescence	
	Xcm[a]	Xcc	XcmA/FITC	Xcca/FITC
X. manihotis (60 isolates)	+	–	+	–
X. cassavae (6 isolates)	–	+	–	+
Colombian yellowish isolates (3 isolates)	–	+	–	+

[a] Xcm = *X. c.* pv. *manihotis*; Xcc = *X. c.* pv. *cassavae*; XcmA/FITC = *X. manihotis* antibody conjugated with Fluorescein isothiocyanate; XccA/FITC = *X. cassavae* antibody labelled with Fluorescein isothiocyanate.

[b] + = positive reaction; – = negative reaction.

antisera produced against *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* was between 2560 and 5120. Some cross-agglutination occurred between *X. c.* pv. *manihotis* and the Colombian yellowish isolates but this was not confirmed by the Ouchterlony double diffusion or by the immunofluorescence tests. No cross-reaction occurred between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* by any of the serodiagnostic methods used (Table 3). However, Immunodiffusion plates showed the presence of antigens common to all the isolates studied (Fig. 1 and 2). In both the double diffusion (Fig. 1 and 2) and immunofluorescence tests, all isolates of *X. c.* pv. *manihotis* were serologically identical and through these tests *X. c.* pv. *manihotis* could be differentiated from *X. c.* pv. *cassavae* and the Colombian yellowish isolates. The Colombian isolates were serologically identical to *X. c.* pv. *cassavae* (Fig. 1) and related to *X. c.* pv. *manihotis*. All *X. c.* pv. *cassavae* isolates were also serologically identical.

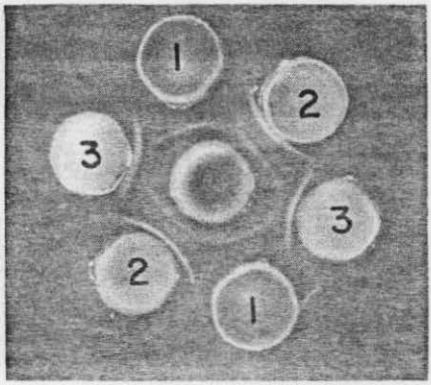

Fig. 1 Central well holds antiserum to *X. c.* pv. *cassavae* (CIAT 1148). Peripheral wells contain: (1) *X. c.* pv. *manihotis* antigen; (2) *X. c.* pv. *cassavae;* (3) Colombian yellowish isolate.

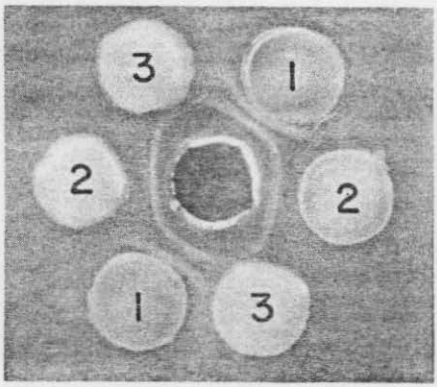

Fig. 2. Central well contains crude antiserum to *X. c.* pv. *manihotis* (CIAT 1105). Peripheral wells: (1) *X. c.* pv. *manihotis* antigen (washed whole cells); (2) *X. c.* pv. *cassavae* antigen (washed whole cells); (3) Colombian yellowish isolate (washed whole cells).

Discussion

Results show that *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* are biochemically similar and serologically related but distinct. This agrees with previous findings (11, 16). While *X. c.* pv. *manihotis* was serologically distinct from *X. c.* pv. *cassavae, X.* pv. *cassavae* and the Colombian yellowish isolates had common antigenic properties, even though differences were found in their pathogenicity. The synonymy between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* reported by Robbs *et al.* (29) was based on biochemical and a few pathogenicity characteristics, but not on serology.

Therefore, the decision to consider *X. cassavae* as a *nomen dubium* (39) is probably unjustified. Based on our results on the serological and pathogenicity relationships between these two pathogens, and their geographical distribution, it is probably unlikely that *X. c.* pv. *cassavae* evolved from *X. c.* pv. *manihotis*. The presence of *X. c.* pv. *cassavae* in Colombia or elsewhere in Latin America therefore remains to be established.

The specificity of our XMA/FITC and XCA/FITC conjugates indicates the high specificity of antiserum produced using glutaraldehyde-fixed whole cells as reported by Allan and Kelman (1) in studies on *Erwinia carotovora* var. *atroseptica*. Similar results have recently been reported (33) in studies with *Corynebacterium sepedonicum*.

Literature Cited

1. Allan, E. and A. Kelman, 1977. Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. Phytopathology 67:1305-1312.
2. Buchanan, R.E. and N.E. Gibbons. 1974. (Eds.) Bergey's Manual of Determinative Bacteriology (8th. Edition). Williams and Wilkins company, Baltimore. 1268 pp.
3. Burkholder, W.H. and M.P. Starr. 1948. The generic and specific characters of phytopathogenic species of *Pseudomonas* and *Xanthomonas*. Phytopathology 38:494-502.
4. Centro Internacional de Agricultura Tropical. Annual Report, 1976.
5. Cherry, W.B. 1974. Immunofluorescence techniques. *In* Manual of Clinical Microbiology 2nd. Ed. pp 29-44. E.H. Lennette, E. H. Spaudling, and J.P. Truant (Eds.). American Society for Microbiology.
6. Christensen, W.B. 1946. Urea decomposition as a means of differentiating proteus and paracolon cultures from Salmonella and Shigella. J. Bacteriol. 52:461-464.
7. Difco Manual. 9th. Ed. 1977. Difco Laboratories, Inc., Detroit Michigan, 350 pp.
8. Dye, D.W. 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas spp.* N.Z.J. Agric. Sci 5:393-416.
9. Dye, D.W. 1968. A taxonomic study of the genus *Erwinia* N.Z.J. Agric. Sci. 11:590-607.
10. Elango, F.N. 1980. Relationship between *Xanthomonas manihotis* (Arthaud-Berthet and Bondar) Starr and *Xanthomonas cassavae* (Wiehe and Dowson), and some epidemiological studies on cassava bacterial blight. Ph.D. thesis, McGill University, Montreal, Canada, 301 pp.
11. Elango, F.N., J.C. Lozano, and J.F. Peterson. 1979. Physiological and Serological relationship between *Xanthomonas manihotis and Xanthomonas cassavae*. (Abstr.). Proc. 5th Symp. Int. Soc. Tropical Root Crops, Manila, Philippines (*In* Press).
12. Hayward, A.C. 1966. Methods of identification in the genus *Xanthomonas* pp. 9-14. In Identification methods for microbiologists. B.M. Gibbs and F.A. Skinner (Eds.). Academic Press, New York.
13. Hildebrand, D.C. 1971. Pectate and pectin gels for differentiation of *Pseudomonas* sp. and other bacterial plant pathogens Phytopathology 61:1430-1436.
14. Hildebrand, D.C. and M.N. Schroth. 1964. B-glucosidase in phythopathogenic bacteria. Appl. Microbiol. 12:487-491.

15. Hugh, R. and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of Carbohydrates by various gram-negative bacteria. J. Bacteriol. 66:24-26.
16. Ikotun, B. 1975. Cassava bacterial blight disease caused by *X. manihotis* (Arthaud-Berthet et Bondar) Starr. Ph.D. thesis, Imperial College of Sci. & Tech., London, 242 pp.
17. Kiraly, Z., Z. Klement, F. Solymosy, and J. Voros. 1974. Methods in Plant Pathology. Elsevier Scientific Publishing Co., New York, 509 pp.
18. King, E.O., M.K. Ward, and D.E. Raney. 1954. Two Simple Media for the demonstration of Pyocyanin and fluorescence. J Lab. Clin. Med. 44:301-307.
19. Klement, Z. 1975. Methods for the separation of closely related bacteria by gel diffusion test. pp 8-10. *In* R.N. Goodman (Ed.). Proceeding of the 1st Workshop on Phytobacteriology (3rd. Ed.) Univ. of Missouri, Columbia. 73pp.
20. Klement, Z., G.L. Farkas and L. Lovrekovich. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54:474-477.
21. Kovacs, N. 1956. Identification on *Pseudomonas pyocyanea* by the Oxidase reaction. Nature (London) 178:703.
22. Lederberg, J. and E.M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol 63:399-406.
23. Lovrekovich, L. and Z. Klement. 1960. Triphenyl tetrazolium chloride tolerance of phytopathogenic bacteria. Phytopath. Z. 39:129-133.
24. Lozano, J.C. 1975. Bacterial Blight of Cassava. PANS 21:38-43.
25. Lozano, J.C. and L. Sequeira, 1974. Bacterial Blight of Cassava in Colombia: etiology. Phytopathology 64:74-82.
26. Maraite, H. and J. Weyns. 1979. Distinctive physiological, biochemical and pathogenic characteristics of *Xanthomonas manihotis* and *Xanthomonas cassavae*. pp. 103-117 *In* H. Maraite and J.A. Meyer (Eds.). Diseases of Tropical food crops, U.C.L. Belgium. 316 pp.
27. Misaghi, I. and R.G. Grogan. 1969. Nutritional and Biochemical comparisons of plant pathogenic and saprophytic fluorescent *Pseudomonads*. Phytopathology 59:1436-1450.
28. Reddy, C.R. and S.H. Ou, 1976. Characterization of *Xanthomonas oryzae* (Uyeda et Ishiyama). Dowson, the bacterial blight pathogen of rice. Ann. Phytopath, Soc. Jpn. 42:124-130.
29. Robbs, C.F., R. de L.D. Ribeiro, O. Kimura, and F. Akiba. 1972. (Variacoes em *Xanthomonas manihotis* (Arthaud-Berthet) Starr. Revista da Sociedade Brasileira de Fitopatologia 5:67-75.
30. Sands, D.C., M.N. Schroth, and D.C. Hildebrand. 1970. Taxonomy of phytopathogenic Pseudomonads. J. Bacteriol. 101:9-23.
31. Sierra, G. 1957. A simple method for the detection of lipolytic activity of microorganisms and some observation of the influence of the contact between cells and fatty substrates. Antonie Van leewenhoek J. Microbial Serology 23:15-22.
32. Skerman, V.B.D. 1967. A guide to the identification of the genera of bacteria (2nd. Ed.) The Williams and Wilkins Co., Baltimore, Maryland. 303 pp.
33. Slack, S.A., A. Kelman and J.B. Perry. 1979. Comparison of three serodiagnostic assays for the detection of *corynebacterium*. Phytopathology 69:186-189.
34. Sneath. P.H.A. 1956. Cultural and biochemical characteristics of the genus *Chromobacterium*. J. Gen. Microbiol. 15:70-98.
35. Society of American Bacteriologists. 1957. Manual of Microbiological Methods. McGraw-Hill Book Co., New York, 315 pp.

36. Starr, M.P. 1979. A concept of the genus *Xanthomonas* and its spp. pp. 393 (Abstr.), *In* Proceedings of the IVth Inter. Conf. on Plant Path. Bact., Angers, France.
37. Terry, E.R. 1978. Cassava Bacterial diseases. pp. 75-84. *In* T. Brekelbaum, A. Bellotti, and J.C. Lozano (eds.). Proceedings Cassava Protection Workshop. CIAT, Cali, Colombia, 7-12 November, 1977.
38. Waksman, S.A. 1967. The Actinomycetes. The Ronald Press Co., New York, 270 pp.
39. Young, J.M., D.W. Dye, J.F. Bradbury, C.G. Panagopoulos, and C.F. Robbs. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N.Z.J. Agric. Res. 21:153-177.

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