

Use of Quinate Metabolism as a Phenotypic Property to Identify Members of *Xanthomonas campestris* DNA Homology Group 6

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ABSTRACT

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Certain pathovars of *Xanthomonas campestris* that metabolize quinate were discovered. This discovery led to the development of a succinate-quininate (SQ) medium for the detection of quinate metabolism. Of five species of *Xanthomonas* and 77 pathovars of *X. campestris*, only members of five pathovars of *X. campestris* were positive in the SQ test. All tested strains of the four pathovars of DNA homology group 6 (i.e., *X. c. celebensis*, *X. c. corylina*, *X. c. juglandis*, and *X. c. pruni*) were positive, except for two strains of *X. c. celebensis*. Strains of *X. c. carotae* also were positive; this pathovar of DNA homology group 5 is most closely related to pathovars of DNA homology group 6. All 100 or more strains

of *X. c. juglandis* isolated from different locations and walnut cultivars were positive, which indicated that this phenotypic property was stable in the population. Analysis of high-performance liquid chromatograms indicated that pathovars positive in the SQ test metabolized quinic acid to gallic acid, protocatechuic acid, pyrogallol, and several unknown compounds. However, most xanthomonads, positive or negative in the SQ tests, cannot utilize quinate as a sole carbon source for growth. These experiments indicated that quinate metabolism but not quinate utilization can be used as a phenotypic property in identifying members of *Xanthomonas* DNA homology group 6.

Additional keyword: taxonomy.

The genus *Xanthomonas* consists mainly of plant-pathogenic bacteria. Only five species were recognized in this genus in the 1980 list and subsequent lists (2). However, one of these, *Xanthomonas campestris* (Pammel) Dowson, has been divided into more than 130 pathovars, each of which supposedly represents a discrete pathogenic entity. This pathovar scheme was adopted only as an interim expedient for retaining the names of previously recognized species that had not been adequately characterized phenotypically (11). As a "special purpose" classification, it does not meet the requirements for naming taxa according to the International Code of Nomenclature of Bacteria (15).

Recent studies have indicated that a number of pathovars can be distinguished by the analysis of fatty acids (13) and restriction fragment length polymorphism (7). Moreover, DNA-DNA homology studies (3,9,15) have indicated that at least six genogroups of pathovars appear to represent distinct species. Nevertheless, these genogroups cannot be recognized as species unless they can be differentiated by various phenotypic properties (16). Accordingly, the search for phenotypic features that distinguish different genogroups is a critical task in the progress of *Xanthomonas* taxonomy. In this report, we describe a test for detecting quinate metabolism; the test distinguishes members of *Xanthomonas* DNA homology group 6, which includes *X. c. celebensis*, *X. c. corylina*, *X. c. juglandis*, and *X. c. pruni* (3,9,15).

MATERIALS AND METHODS

Bacterial strains. Xanthomonads were obtained from the International Collection of Phytopathogenic Bacteria, the Department of Plant Pathology, University of California, Berkeley, and the American Type Culture Collection. The species and numbers of xanthomonads were *X. albilineans* (one strain), *X. axonopodis* (one strain), *X. campestris* (342 strains), *X. fragariae* (three strains), and *X. malitiphilia* (one strain). Strains of *X. campestris*, including type strains, were from 77 pathovars and represent all six genetic groups and several ungrouped pathovars (3,9,15). The number in the parentheses represents the number of tested strains of each pathovar, and the asterisk

indicates that the strains tested include the type strain. DNA homology group 1 includes *alfalfae* (2), *amaranthicola** (1), *begoniae** (6), *betlicola** (2), *biophyti** (1), *blepharidis** (1), *cajani** (1), *cassiae** (1), *coriandri** (1), *fascicularis** (1), *glycines** (8), *khayae** (1), *lawsoniae** (1), *lespedezae** (2), *maculifoliogardeniae** (2), *malvacearum* (7), *mangiferaeindicae* (1), *martyniicola** (1), *melhusii** (1), *musacearum** (1), *naka-taeorchori** (1), *patelii** (2), *phaseoli** (16), *phaseoli* var. *fuscans** (7), *physalidicola** (1), *physalidis** (1), *poinsettiiicola** (6), *ricini** (1), *tamarindi** (2), *vignicola** (7), *vitians** (3), *vitiswoodrowii** (1). DNA homology group 2 includes *armoraciae** (1), *campestris* (9), *incanae** (6), *plantaginis** (4), *raphani** (2). DNA homology group 3 includes *arrhenatheri** (1), *cerealis* (4), *graminis** (1), *hordei* (3), *phlei** (1), *poae** (1), *secalis** (1), *translucens* (2), *undulosa** (4). DNA homology group 4 includes *oryzae** (3), *oryzicola** (6). DNA homology group 5 includes *carotae** (4), *gardneri** (1), *pelargonii** (9), *taraxaci** (1). DNA homology group 6 includes *celebensis** (3), *corylina** (7), *juglandis** (114), *pruni** (13). Others are *badrii** (1), *clerodendri** (1), *desmodii** (1), *desmodiigangetici** (1), *dieffenbachiae* (5), *erythrinae** (1), *eucalypti** (2), *hederae* (1), *holcicola** (2), *hyacinthi** (1), *manihotis** (11), *nigromaculans** (1), *papavericola** (3), *pisi** (2), *punicae** (2), *sesbaniae** (1), *spermacoces** (1), *trichodesmae** (1), *uppalii** (1), *vasculorum** (6), *vesicatoria** (6).

More than 100 recently isolated *X. c. juglandis* strains were tested. These strains were isolated from buds and twig cankers of different walnut cultivars grown in California by using brilliant cresyl blue starch (BS) semi-selective medium (8). The pathogenicity of strains was tested with a Carborundum inoculation method on walnut seedlings grown in the greenhouse.

Quinate metabolism test. Numerous trials resulted in the development of a succinate-quininate (SQ) medium, which reliably detected the metabolism of quinate in xanthomonads of group 6. The SQ medium (per liter) was composed of 10 g of succinic acid (disodium salt, hexahydrate), 5 g of quinic acid, 1.5 g of K₂HPO₄, 1.0 g of (NH₄)₂SO₄, and 15 g of agar. The pH was adjusted to 7.2-7.5 with 10 N NaOH and autoclaved for 20 min at 121 C. The medium was cooled to about 50 C, and then 7.5 ml of autoclaved 20% MgSO₄·7H₂O solution was added. For some xanthomonads that grew poorly on SQ medium, 0.5 g/L of yeast extract was added to the medium.

Four to six bacterial cultures per plate were streaked and incubated for 4–6 days at 28 C. The diffusion of a deep-green around a bacterial streak was considered a positive reaction. The test for each strain was repeated at least three times.

Carbon source utilization. The capacity of xanthomonads to utilize various aromatic compounds for growth was tested with the basal medium of Ayers et al (1) supplemented with 0.1% of a carbon source and occasionally 0.25 g/L of yeast extract. The aromatic compounds tested included caffeic acid, gallic acid, phenylalanine, protocatechuic acid, quinic acid, shikimic acid, and tyrosine. Growth or no growth of inoculated bacteria was scored after 7 and 14 days of incubation at 28 C.

Metabolite analysis. Several SQ positive and negative strains were used for metabolite analysis. The bacteria were inoculated into 5 ml of SQ broth and incubated at 28 C for 4–5 days with shaking. The bacterial cells were removed by centrifugation at 14,000 rpm in microfuge tubes for 10 min. The supernatants were acidified with 1 N HCl to pH 2.0, then freeze-dried, and dissolved in methanol. The methanol solution was analyzed with high-performance liquid chromatography (HPLC).

HPLC was performed with an LC pump (Perkin-Elmer, series 410; Norwalk, CT) and a Perkin-Elmer LC-95 UV/visible spectrophotometric detector operated at 250 nm. The HPLC column (250 × 4.6 mm i.d., Microsorb) packed with 5 μm of C₁₈-bonded reversed-phase material was obtained from Rainin Instrument Co. Inc. (Emeryville, CA). The mobile phase was 0.01 M ammonium dihydrogen phosphate made to pH 2.6 with phosphoric acid and run at a flow rate of 1.5 ml/min (14). Quinic acid, gallic acid, pyrogallol, and protocatechuic acid used to prepare the standard solutions are commercial products from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Quinate metabolism detection. Xanthomonads positive for the SQ test were detected only in *X. campestris* DNA homology groups 5 and 6. All strains of the four pathovars of DNA homology group 6, *celebensis*, *corylina*, *juglandis*, and *pruni*, were positive except for two of the three strains of pathovar *celebensis* (XC143 and XC144). XC144, however, produced a reddish color in this test. Strains of *X. c. carotae*, the pathovar of DNA homology group 5 that is most closely related to pathovars of DNA homology group 6 (9), also were positive. More than 100 strains of *X. c. juglandis* isolated from walnut cultivars in different locations were positive. This indicated that this phenotypic property was stable in the population of *X. c. juglandis*.

Growth on aromatic substrates. Xanthomonads cannot utilize quinate or shikimate as a sole carbon source for growth, with the exception of one strain of *X. c. celebensis* (XC144) and one strain of *X. fragariae* (UCPPB 861). Both of these strains gave a negative reaction in the SQ test, although as noted, XC144 produced a reddish color in the SQ test. Caffeic, gallic, and protocatechuic acids cannot be used for growth, and utilization of phenylalanine and tyrosine is varied in xanthomonads.

HPLC analysis. Analysis of HPLC chromatograms indicated that pathovars positive in the SQ test metabolize quinic acid and that negative pathovars lack this ability. Gallic acid was identified as one of the major metabolites. Other metabolites identified were pyrogallol and protocatechuic acid. Several additional metabolites were observed but have not been identified. Some of these may be auto-oxidation products of gallic acid, such as digallic, trigallic, and ellagic acids (14). The patterns of HPLC chromatograms of all positive pathovars were the same, suggesting that positive pathovars utilize the same biochemical pathway to metabolize quinate. Xanthomonads that were negative in the SQ test did not accumulate these compounds. The HPLC patterns of *X. c. celebensis* strain XC144, which was negative in the SQ test but utilized quinate for growth, were different from those of SQ-positive or other SQ-negative strains (data not shown).

Effect of other carbon sources on color development. When carbon sources such as glucose, fructose, galactose, sucrose, lactose, maltose, and glycerol were substituted for succinate in

the SQ medium, there was no deep-green around the bacterial streak, which is an indication of the breakdown of quinate. Color development occurred only when succinate or citrate was used. Analysis of HPLC chromatograms showed that quinate metabolism was not repressed by carbon sources such as glucose and fructose. One of the reasons for the failure of color development may be the pH values. The pH in media with succinate or citrate as the carbon source is higher, about 9.5, after 4–5 days of incubation. The pH value with glucose or fructose, however, is about 4.5. Because gallic acid is a deep-green above pH 7, the color development in the SQ medium may be the result of the accumulation of gallic acid and related products from quinic acid.

DISCUSSION

The nearly universal expression of quinate metabolism among xanthomonads of DNA homology group 6 and its general absence among other xanthomonads makes the SQ test very useful for the identification and delineation of the DNA homology group 6. The other xanthomonads that expressed quinate metabolism, *X. c. carotae* and one strain of *X. fragariae*, can be differentiated from members of group 6 on the basis of other tests. For example, *X. c. carotae* does not grow on the BS medium (8) because it cannot utilize starch. Consequently, strains isolated on the BS medium that express quinate metabolism on the SQ medium should almost always belong to group 6.

Although xanthomonads in group 6 can metabolize quinate to gallic acid, they cannot utilize quinate or gallic acid for growth. This points out limitations in using growth and nutritional tests for defining phenotypic properties because the tests do not detect utilization of substrates for purposes other than growth. This nongrowth mode of utilization also occurs with the pseudomonad β-glucosidase system. *Pseudomonas syringae* hydrolyzes glucosides such as arbutin and salicin but does not use the products for growth (5). Because quinates and glucosides exist in a variety of plants, it would be useful to know why they are metabolized. These metabolic pathways may relate to such activities as survival and pathogenesis, or they may simply be the result of loss of important enzymes during the course of evolution. Such enzymes are needed for further conversion of substrates to compounds that can be utilized for growth.

Some bacteria degrade quinic acid or shikimic acid via the hydro-aromatic pathway to protocatechuic acid and further by the β-ketoadipate route to succinate and acetyl-CoA (4,6). Most fluorescent pseudomonads such as *P. s. syringae* have these biochemical pathways and can utilize quinate for growth (10,12). These pseudomonads produce a color change (yellowish) on the SQ medium. Analysis with HPLC, however, did not detect gallic acid, but rather indicated that protocatechuic acid was the major product that accumulates (data not shown). This would suggest that the metabolic pathway for quinate breakdown by xanthomonads in group 6 is different from that of the fluorescent pseudomonads. Further evidence of this difference comes from *X. c. celebensis* XC144. Although this strain can utilize quinate for growth like pseudomonads, the compounds accumulated are quite different from those accumulated by fluorescent pseudomonads (data not shown). To our knowledge, the bacterial biochemical pathway from quinic acid to gallic acid, which is a precursor of gallotannin, has not been reported. Therefore, the understanding of this pathway in the pathogenic xanthomonads should be an important approach to exploring the biological functions and biochemical pathway of quinate metabolism.

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