*European Journal of Plant Pathology* **103:** 687–693, 1997.



# Differentiation of three species of *Xanthomonas* and *Stenotrophomonas maltophilia* using cellular fatty acid analyses *

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Accepted 10 July 1997

*Key words:* classification, FAME, phytobacteria

**Abstract**

Five hundred eighty-eight strains, representing *Xanthomonas albilineans, X. fragariae,* ten pathovars of *X. campestris,* and *Stenotrophomonas maltophilia* from ornamentals, were subjected to fatty acid methyl ester (FAME) analyses. Quantitative variance among FAME profiles enabled identification of the four species with 100% accuracy. Dendrogram cluster analysis placed strains of *X. albilineans* remotely from those of the other two*Xanthomonas* species and *S. maltophilia*. Whereas some profiles of pathovars of *X. campestris* were distinct, strains within *X. albilineans, X. fragariae,* and *S. maltophilia* were homogeneous by their conserved FAME ratios. Pathovars of *X. campestris* that had conserved profiles were*fittonia, hederae, malvacearum, pelargonii,* and *zinniae*. FAME profiles of *X. campestris* pathovars *begoniae, dieffenbachiae, fici, maculifoliigardeniae,* and *poinsettiicola* were, however, quantitatively diverse. These pathovars did not form discrete subgroups, and intercalated randomly with one another on the dendrogram. Certain species or pathovars of *X. campestris* which have homogeneous FAME profiles can easily be identified with fatty acid analysis; however, pathovars of *X. campestris* with heterogeneous profiles are not readily identified by fatty acid analysis.

## Introduction

A number of biochemical and physiological tests have been used by researchers to identify xanthomonads. Most researchers, before 1980, relied on the methods used by Dye (Dye, 1962) and on host specificity to characterize xanthomonads. Physiological and bacteriological characteristics of many nomenspecies in the genus *Xanthomonas* were insufficient to quantify these bacteria as 'species.' Hence, many pathovars were grouped into a single species '*X. campestris*' and pathovar names have been given according to host of origin and previously published research on closely related hosts. Wernham tested the pathogenicity of 14 strains of *X. campestris* on 12 hosts and found a very high degree of host specificity (Wernham, 1948). Additional research by Dye (1958), based on inoculation of *Phaseolus vulgaris* L. (bean) with 20 strains of different *X. campestris* pathovars, indicated that host specificity was not present. However, in 1966, Schnathorst (1966) reported unaltered host specificity of strains passed through beans. Leyns et al. (1984) published a comprehensive review of host range studies on *Xanthomonas*; some strains were strongly host specific, others had a wide host range. Those authors suggested that DNA-DNA homology in combination with numerical analysis of morphological, physiological and biochemical tests of a large number of strains should be used to identify strain groups, or pathovars, of *X. campestris*.

Insufficient host range testing in studies has resulted in a proliferation of pathovar names. Additional confusion in classification may occur due to the reliance on type cultures which have not been selected to properly characterize a given pathovar. In 1980, a listing of accepted pathovar names, as well as standards to

* Florida Agricultural Experiment Station Journal Series No. R-05008

be used for naming pathovars of phytopathogenic bacteria, was published to establish the validity of the growing list (Dye et al., 1980).

During the past 10 to 20 years it has become increasingly apparent that other methods must be sought to identify species of *Xanthomonas* as well as pathovars of *X. campestris*. Recently, a number of papers have been published which evaluated monoclonal antibodies (Alvarez et al., 1985; Lipp et al., 1992), restriction fragment-length polymorphism analysis (Lazo and Gabriel, 1987; Lazo et al., 1987), fatty acid analysis (Chase et al., 1992; Roy, 1988; Stead, 1989), genomic DNA fingerprinting (Cooksey and Graham, 1989), plasmid DNA fingerprinting (Pruvost, 1992), 16S rRNA sequences (Deparasis and Roth, 1990), and DNA homology (Grimont, 1988; Hildebrand et al., 1982; Hildebrand et al., 1990; Murata and Starr, 1973) for usefulness in characterizing single pathovars of *X. campestris* or groups of strains from a number of pathovars. Researchers in several comprehensive studies have evaluated a large number of assays to numerically analyze *Xanthomonas* spp. (Van den Mooter and Swings, 1990; Vauterin et al., 1990a; Vauterin et al., 1990b; Vauterin et al., 1991). Widespread use of some of these methods is limited, however, due to expense, time, spectrum of use, or ability to differentiate strains at the pathovar level.

A recent DNA-DNA hybridization study by Vauterin et al. (1995), on 183 strains of *Xanthomonas,* has resulted in the proposal of 20 DNA homology groups which are considered to be genomic species. Four of the 20 DNA homology groups are previously described species: *X. albilineans, X. fragariae, X. oryzae,* and *X. populi.* Vauterin et al. divided *X. campestris* into 16 DNA homology groups, and proposed a comprehensive revision of the classification of the genus *Xanthomonas.*

One of the most promising methods which minimizes cost and time, and enables differentiation among a broad spectrum of strains (plant pathogenic and many saprophytic bacteria), is cellular fatty acid analysis (Sasser, 1990; Welch, 1991). Many studies have indicated potential use within a pathovar group to characterize strains, but no study has been published which compared a large number of species of *Xanthomonas* as well as pathovars of *X. campestris*. The objective of this research was to evaluate fatty acid analyses for identification of *S. maltophilia,* and three species of *Xanthomonas,* including ten pathovars of *X. campestris*.

*Stenotrophomonas maltophilia* was first named and described by Hugh and Ryschenkow (1960, 1961) as *Pseudomonas maltophilia*. This species of bacteria is characterized as being gram-negative, motile by polar multitrichous flagella, yellow pigmented (not xanthomonadin), with a limited ability to utilize many organic compounds. *S. maltophilia* is not considered to be a plant pathogen; although frequently isolated from plant material. However it is an important opportunistic nosocomial pathogen of humans (Schable et al., 1992). Hugh and Ryschenkow (1961) initially described 15 serotypes of *S. maltophilia*. This number was later expanded by Schable et al. (1989) to twenty-six serotypes. Using these serotypes, it has been possible to conduct epidemiological surveys of strains found in the environment and from medical isolates (Schable et al., 1992). Palleroni et al. (1973) using hybridization techniques compared rRNA cistrons of the then *Pseudomonas maltophilia* to *Xanthomonas* spp showing a genetic relationship between these genera. This genetic relationship was further confirmed by Swings et al. (1983) using DNA-rRNA hybridization. A similarity coefficient of 75% between *P. maltophilia* and *Xanthomonas* spp. was calculated from these results. Based on these findings, it was proposed that the genus name be changed to *Xanthomonas*. More recently, Palleroni and Bradbury (1993) have proposed a separate genus, *Stenotrophomonas.* This decision was primarily based on the many physiological and carbon source utilization differences of this genus to those of *Xanthomonas* spp. Strains of *S. maltophilia* were added to this study to further examine the relatedness of these bacteria to *Xanthomonas.*

## Materials and methods

*FAME extraction.* Because the growth rate of many of the strains in this study was too slow to allow development of single colonies within the Microbial ID Inc. (MIDI) -recommended 24 h time period, it was decided *a priori* to extract cells grown for a longer time. With the exception of Yeast Sucrose Peptone medium (Dye, 1962) for *X. albilineans,* and Wilbrink's medium (Ricaud and Ryan, 1989) for *X. fragariae,* all strains were grown on Difco Trypticase Soy Agar amended with 5% sucrose (TSA) for 48 h at 28 ($\pm$) 1 °C. Each sample was quadrant streaked, and cells in the late log phase of growth (third quadrant) were harvested 48 h after inoculation. Approximately 40 mg cells (wet weight) were transferred to 13 $\times$ 100 mm glass tubes fitted with Teflon-lined screw caps and fatty acids were extracted using methods described by Miller (1982). In

*Table 1.* Source of strains of *Xanthomonas* used in FAME analysis

| Strain identification | No. strains | Laboratory source |
|---|---|---|
| *X. albilineans* | 17 | 6 |
| *X. campestris* | | |
| pv. *begoniae* | 66 | 2, 3, 4, 10, 12, 13 |
| pv. *dieffenbachiae* | 166 | 1, 2, 3, 7, 10, 11, 12, 14 |
| pv. *fici* | 26 | 3, 10, 12, 13 |
| pv. *fittonia* | 24 | 2, 3 |
| pv. *maculifoliigardeniae* | 25 | 2, 3, 10, 12, 15 |
| pv. *malvacearum* | 26 | 2, 3, 10, 12 |
| pv. *pelargonii* | 140 | 1, 2, 3, 5, 8, 10, 12, 13, 14 |
| pv. *poinsettiicola* | 33 | 1, 2, 3, 8, 10, 12, 13, 15 |
| pv. *zinniae* | 36 | 3, 10, 12, 13, 14, 15 |
| *X. fragariae* | 10 | 2, 3, 10 |
| *S. maltophilia* | 19 | 3, 5, 9, 13 |

Strains were supplied by the following laboratories: (1) A. Alvarez, Dept. of Plant Pathology, University of Hawaii at Manoa, Honolulu, HI 96822; (2) D. Brunk, Plant Disease Diagnostics, Inc. Apopka, FL 32703; (3) A. R. Chase, Dept. of Plant Pathology, University of Florida, CFREC, Apopka, FL 32703; (4) D. A. Cooksey, Plant Pathology Dept., University of California, Riverside, CA 92521; (5) M. Daughtrey, Long Island Horticultural Research Laboratory, Riverhead, NY 11901; (6) M. Davis, Dept. of Plant Pathology, University of Florida, 18905 SW 280th Street, Tropical Res. Education Ctr., Homestead, FL 33031; (7) R. Dickey, Dept. of Plant Pathology, Cornell University, Ithaca, NY 14853; (8) J. B. Jones, Dept. of Plant Pathology, University of Florida, 5007 60th Street East, Bradenton, FL 34203; (9) D. T. Kaplan, USDA-ARS, 2120 Camden Rd., Orlando, FL 32803; (10) J. Miller, Division of Plant Industry, Florida Dept. of Agriculture and Consumer Services, Gainesville, FL 32602; (11) K. Pohronezny, University of Florida, Tropical Research and Education Center, Homestead, FL 33031; (12) G. W. Simone, Dept. of Plant Pathology, University of Florida, Gainesville, FL 32611; (13) R. E. Stall, Dept. of Plant Pathology, University of Florida, Fifield Hall, Gainesville, FL 32611; and (14) Yoder Bros. Inc., Alva, FL 33920; (15) J. M. Young, D.S.I.R., MT Albert Research Centre, Private Bag, Auckland, New Zealand.

order to compare the 24 h FAME profiles with those obtained at 48 h, a subset of the strains tested were compared by harvesting 24 h growth from multiple petri plates of the same media. No new fatty acids were detected among the 48 h strains, but relative ratios of the fatty acids differed slightly.

*Library generation.* A separate library, comprised solely of the strains grown for 48 h, was created using the MIDI Library Generation System (LGS) program, to compensate for the altered growth time. The LGS software enables a user to identify and compare fastidious or slow-growing bacteria which cannot be cultured using standard MIDI conditions. To determine the utility of fatty acid analysis as an assay for identification of xanthomonads at the pathovar level, a library was created from FAME profiles of 588 strains isolated from a variety of ornamental plants (Table 1). FAME profiles of unknown strains could then be classified against the 48 h library, and closest matches could be statistically determined by the MIDI software program.

*Strain classification.* Fatty acid profiles from a 588-member sample population were initially compiled for numerical analysis. A single library entry was created using the MIDI Library Generation System software, which statistically compares qualitative and quantitative aspects of each strain's FAME profile. The library entry included the mean (x), standard deviation ($\sigma$), and variance (x/$\sigma$) for each acid detected in the population. The MIDI '10% Rule' states that an acid having a [x/$\sigma$] greater than 0.10 should be histogram-edited, and the corresponding strains placed into subgroups (Mendala, 1990). Each block on the histogram of a fatty acid represents one strain in the population. The location of a block on the histrogram corresponds to the amount of that acid detected in the strain. Thus, strains having the highest or lowest amount of the histogrammed fatty acid will appear as an outlier on the far right or left, respectively, of the population. The acid present in all strains in the population (15:0 *iso*) was histogrammed first.

On the 15:0 *iso* histogram, all *X. albilineans* strains segregated conspicuously from the population because of significantly lower quantities of 15:0 *iso* ($\sim$5%) were detected in those strains (Table 1). Histogram-editing was continued, using 15:0 *anteiso*, 16:1 $\omega$ *cis* 9, and 16:0, respectively, until the (x/$\sigma$) for the majority of the fatty acids in the library entry profile was below 0.10, and all of the 588 strains were placed in subgroups. Because profiles of some pathovars e.g., *X. c.* pv. *dieffenbachiae*, and *begoniae*, were heterogeneous, compliance with the MIDI '10% Rule' necessitated creating several library entry subgroups. For example, nine subgroups were required for the strains from *dieffenbachiae* (Chase et al., 1992).

Variation in levels of hydroxy acids is useful in the identification of many pathovars of *X. campestris* (Roy, 1988). Several hydroxy acids accounted for the heterogeneity of *X. c.* pv. *dieffenbachiae*, *begoniae*, *poinsettiaecola*, and *fici* strains, e.g., 11:0 2-OH, 13:0 2-OH, and 16:0 2-OH. Applications of the MIDI '10% Rule' caused the 588-member population to be placed into 29 entries in the 48 h library. The utility of the library generation was to create a database of phytopathogenic

xanthomonads, and use that library to identify FAME profiles of unknown xanthomonads from ornamentals.

*Dendrogram generation.* Using the MIDI dendrogram program, relationships among members of a population were graphically portrayed, based upon the qualitative and quantitative aspects of sample profiles. A 12-page dendrogram of the 588-member population was constructed; the need for a less cumbersome version was apparent. To condense the large sample population while preserving interrelationships, three representatives from each *X. campestris* pathovar, or species of *Xanthomonas*, were selected using the LGS software. Although some pathovars of *X. campestris* (e.g. *dieffenbachiae*) had been placed in several library subgroups for future classification and identification of unknown *xanthomonads*, the 10 pathovars were regrouped and three individual strains from each pathovar were selected using the LGS distance histrogram function. Unlike histograms of individual fatty acids, distance histograms portray a population as a function of its total fatty acid profile. Each block on a distance histogram represents an individual strain; distance units are standard deviations, from the mean of all of the fatty acids in the population. Separate distance histograms were created for strains of *X. albilineans*, *X. fragariae*, *S. maltophilia*, and each of the 10 pathovars of *X. campestris*. The three samples located closest to the mean (centroids) on each of the 13 distance histograms were selected; thus, the 588-member population was depicted on a simplified, representative dendrogram using 39 strains.

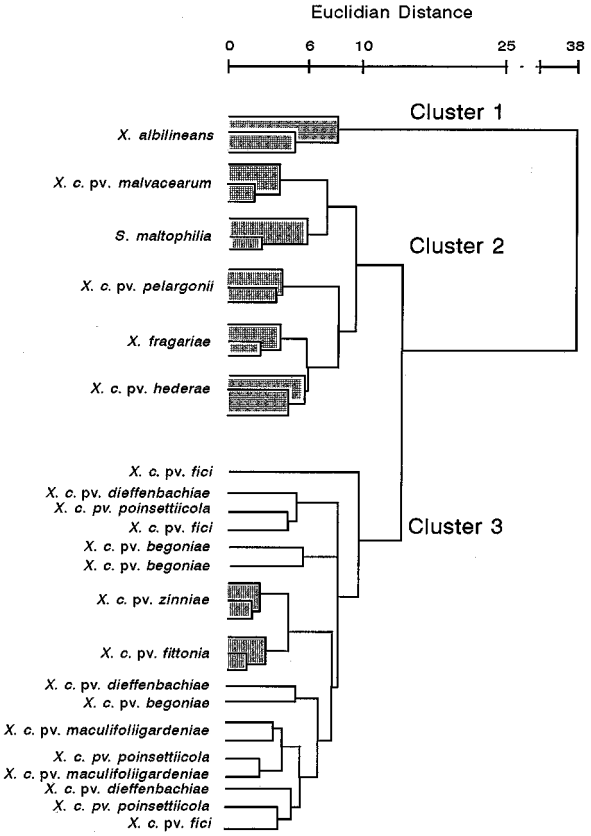


*Figure 1.* Dendogram cluster analysis based on FAME profiles of 588 strains of *X. albilineans, X. campestris, X. fragariae,* and *S. maltophilia.*

## Results and discussion

FAME profiles of xanthomonads are complex and consist of 18–25 fatty acids. Four fatty acids comprise 35–60% of the total profile: 15:0 iso, 15:0 *anteiso*, 16:0, and 16:1 $\omega$ cis 7. Quantitative variation of these fatty acids facilitates rapid and accurate differentiation among species of *Xanthomonas* (Chase et al., 1992). The MIDI pattern recognition software utilizes qualitative and quantitative aspects of FAME profiles, and ratios of related fatty acids (e.g., the structural isomers, 15:0 iso and 15:0 *anteiso*), to assess similarities among members of a population. Unweighted pair matchings, based on FAME profiles, were created by MIDI cluster analysis algorithms (Sasser, 1990a). Comparisons of strains can be presented as a dendrogram (Figure 1), which portrays taxonomic relationships among a group of pre-selected organisms. Based upon fatty acid profiles, samples linking at specified Euclidian Distance (ED) units have the following relationships: strains linking at $\leq$ 25 ED units are likely to belong to the same genus; those linking at ED $<$ 10 or $<$ 6 are likely to be the same species, or biotype, respectively (Sasser, 1990b). A histogram of the predominant acid, 15:0 iso, revealed four outlying clusters, three of which contained significantly higher percentages of 15:0 iso than the population mean, and one which had significantly lower levels of that acid. Each cluster was edited, assigned a numerical suffix, and removed from the population. All of the samples in the cluster which had the highest amount ($\sim$35%) of 15:0 iso were strains of S. *maltophilia.* All of the strains in the adjacent cluster (15:0 iso = $\sim$33%) were *X c*. pv. *malvacearum;* the third cluster was comprised exclusively of *X. fragariae* (15:0 iso = $\sim$26%). Strains in the subgroup on the far left of the histogram contained a much lower level of 15:0 iso ($\sim$3%). All of the members of that group were

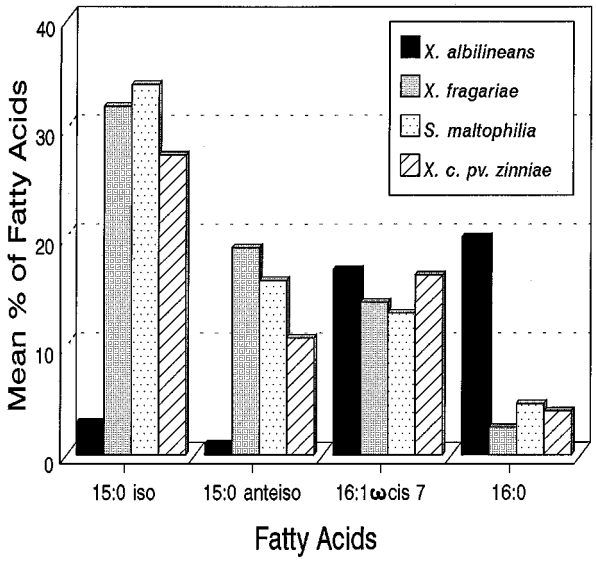


*Figure 2.* Percentages of four major fatty acids in the FAME profiles of *X. albilineans, X. fragariae, S. maltophilia* and *X. c.* pv. *zinniae.* The four fatty acids comprise 35–60% of the total profile, and can be used to differentiate species of *Xanthomonas*.

determined to be *X. albilineans.* The larger distance, 38 ED units, indicates that *X. albilineans* may represent a separate genus (Figure 1). Vauterin et al. recently proposed a new species, *X. sacchari*, for strains recovered from diseased sugarcane in Guadeloupe based on distinct protein and FAME profiles from those of typical *X. albilineans* (Vauterin et al., 1992).

FAME profiles of *X. albilineans* are unlike those of *X. campestris* pathovars. Two characteristic fatty acids of xanthomonads are 15:0 iso and 15:0 anteiso [Myron Sasser, pers. comm.]. Quantitative factors which enable the differentiation of species of xanthomonads include low levels of 15:0 iso, and high levels of several 16- and 17-carbon fatty acids detected in *X. albilineans*. An important qualitative difference was the trace levels of 15:0 anteiso found in *X. albilineans* strains used in this study. Percentages of four major fatty acids in the FAME profiles of *X. albilineans, X. fragariae, S. maltophilia,* and a representative *X. campestris* (pv. *zinniae*), are presented in Figure 2. The current MIDI library database (TSBA version 3.80) includes three species of *Xanthomonas* in which 15:0 anteiso is either not detected, or is present in trace amounts as, for example, in *X. albilineans, X. axonopodis,* and *X. oryzae* (MIDI, 1993). All samples in the second dendrogram cluster grouped uniformly, and with no integration of strains from other species or pathovars. For example, the three strains representing *X. c.* pv. *malvacearum* formed one homogeneous subgroup which linked, at an ED $< 10$, to another subgroup that contained only strains of *S. maltophilia*. The current taxonomic designation of species of *Xanthomonas* may need re-examination since *S. maltophilia* and *X. malvacearum* fall within the species concept ($< 10$ ED units), using fatty acid analyses as the sole criterion.

The three remaining subgroups in the second cluster were comprised exclusively of strains of (1) *X. campestris* pv. *pelargonii* (2) *X. fragariae* and (3) *X. c.* pv. *hederae*. All members of the second cluster exhibited homogeneous, conserved FAME profiles which consistently remained within the MIDI '10% Rule'. The species in the second cluster were: *X. albilineans, X. fragariae,* and *S. maltophilia*. Some pathovars of *X. campestris* also demonstrated conserved FAME profiles (*i.e., hederae, malvacearum*, and *pelargonii*).

The third dendrogram cluster consisted solely of pathovars of *X. campestris*. Members of this cluster intercalated randomly with each other, with the exception of two homogeneous subgroups: *X. c.* pv. *fittonia* and *zinniae*. Despite their location on the dendrogram with heterogeneous *X. c.* pathovars, the *X. c.* pv. *zinniae* and *fittonia* subgroup produced uniform FAME profiles, both of which exhibited very little variance. The FAME profiles of all other pathovars in the third cluster were quantitatively diverse, and library entry profiles for those pathovars consistently exceeded the MIDI '10% rule'. Heterogeneous pathovars of *X. campestris,* which intercalated randomly with each other on the dendrogram, include: *begoniae, dieffenbachiae, fici, maculifoliigardeniae,* and *poinsettiicola*.

*X. albilineans* and *X. fragariae, S. maltophilia,* and some pathovars of *X. campestris* e.g., *hederae, fittonia, malvacearum, pelargonii* and *zinniae* exhibited homogeneous, conserved fatty acid profiles. Library entry profiles for those strains consistently remained within the MIDI '10% rule', substantiating the observed low variance. Other *X. campestris* pathovars, (e.g., *begoniae, dieffenbachiae, fici, maculifoliigardeniae,* and *poinsettiicola*) produced quantitatively diverse profiles which consistently exceeded the '10% rule'.

This work has demonstrated that FAME analyses can be used most effectively to identify groups of xanthomonads which are homogeneous with respect to fatty acid composition. In order to conduct a comprehensive taxonomic study of the genus *Xanthomonas,* analyses should include FAME profiles, rRNA restriction patterns, DNA-DNA hybridization groupings,

phenotypic traits, carbon substrate utilization, SDS-PAGE of proteins, and host specificity.

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