WHOLE CELL FATTY ACID ANALYSIS AS A TOOL FOR CLASSIFICATION OF PHYTOPATHOGENIC PSEUDOMONAS BACTERIA

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Proefschrift
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doctor in de landbouw- en milieuwetenschappen,
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Aan: Anneke
Monika, Margriet, Jaap en Hans

STELLINGEN

1. Dat er zonder de vraag om of noodzaak van identificatie geen classificatie of nomenclatuur en dus ook geen taxonomen nodig zijn (C.T. Cowan, A dictionary of microbial taxonomy, Cambridge University Press, 1978), wordt door veel taxonomen te weinig onderkend.

2. In tegenstelling tot de situatie in de virologie kan de serologie in de (fyto)bacteriologie slechts een hulpmiddel zijn bij detectie en identificatie. Hoewel er gunstige perspectieven lijken te zijn, is nog lang niet bekend in hoeverre de nucleïnezuurtechnologie hier uitkomst brengt.

3. Het typeren als een fylogenetische kleuring van de techniek, waarbij bacteriën zichtbaar gemaakt worden op een microscoopglaasje door hybridisatie met fluoresceïne-gemarkeerde oligonucleotiden, is een misvatting.

   E.F. De Long & al., Science 243: 1360-1363, 1989.

4. De vertaling van de naam 'Pseudomonas' als 'foutieve eenheid' is te simpel.

   C.R. Woese & al., Syst. appl. Microbiol. 5: 179-195 (1984). Dit proefschrift.

5. Dat diagnostiek van ziekten en plagen zou kunnen verlopen via een computer en kunstmatige intelligentieprogramma's, zonder inschakeling van de initiërende en interpreterende kennis en ervaring, opgeslagen in de hersenen van experts, is een fictie.

   A.H.C. van Bruggen & al., Plant Disease 75: 320-322, 1991

6. Harmonisatie op het gebied van de wetgeving rond pathogene quarantaine organismen kan worden versneld door het instellen van expertgroepen en het financieren van gezamenlijke onderzoekprogramma's van deze groepen in EG-verband.

CONTENTS

Fig. 1. *Pseudomonas syringae* NCPPB 981 showing polar flagella, in normal light microscopy. Silver stain according to M.E. Rhodes, J. gen. Microbiol. 18: 639-648 (1958). Magnification c. 5000x

'Woll wir durch Lernung unser Vernunft schärpfen und uns doss einüben, so mügen wir wohl etlich Wahrheit durch recht Weg suchen, lernen, erkennen, erlangen und dazu kommen'.

Albrecht Dürer

I. GENERAL INTRODUCTION

I.1. Outline of this study

Many plant pathogenic bacteria belong to the genus *Pseudomonas*. They have a world wide distribution and are found associated with most major groups of higher plants. Species like *P. syringae* and *P. solanacearum* are primary pathogens and cause devastating diseases in many economically important crops. Others like the soft rot pseudomonads[1)] are opportunistic pathogens and are especially important in post harvest crop losses (Bradbury, 1986; Schroth et al., 1983). The taxonomy of the phytopathogenic (and other) pseudomonads is in many cases unclear and unsettled. This is due to: 1) insufficient description in the past, 2) changes in taxonomic concepts, 3) contradictory data and 4), for the phytopathogens, difficulties in establishing and estimating the value of pathogenicity in taxonomy (Palleroni, 1984; Burkholder & Starr, 1948). To clarify taxonomic problems, especially those related to 4) I studied some representatives of the phytopathogenic pseudomonads. Both conventional morphological, physiological, biochemical and pathogenicity tests and a relatively new 'finger-print' technique, namely fatty acid analysis, were applied.

The taxonomy of the genus *Pseudomonas*, including the phytopathogens, in a historical perspective is outlined in chapter I.2. Here it is enough to say that the genus *Pseudomonas* comprises aerobic Gram-negative, rod-shaped, non-sporeforming bacterial species with a respiratory metabolism and polar flagella (Fig. 1). On the basis of DNA-r(ibosomal)RNA hybridizations, the genus has been divided in at least four homology groups (Palleroni et al., 1973). Plant pathogenic species such as *P. syringae* and the soft rot pseudomonads which show diffusible fluorescent pigments in culture are found in rRNA group I, the so called *P. fluorescens* homology group. The non-fluorescent species, like *P. solanacearum* are found in rRNA group II, the so called *P. pseudomallei-P. cepacia* homology group.

[1)] 'pseudomonad ' is a commonly used term for a bacterial strain belonging to the genus *Pseudomonas*.

In the past a narrow species concept has been used for the fluorescent phytopathogenic pseudomonads. A species rank was primarily based on the host plant attacked and the nature of the symptoms evoked. Some of these (nomen)species appeared to be identical to *P. syringae*, a wide host- range pathogen, or to differ from this species in host range and pathogenicity only. Because some nomenspecies were difficult to identify using bacteriological tests then in vogue Doudoroff and Palleroni (1974), in Bergey's Manual of Determinative Bacteriology, only distinguished *P. syringae, P. cichorii and P. fluorescens* (some strains of biovar 2) as valid fluorescent pathogenic species. The pathogenic individuality of the nomenspecies, however, was completely lost. From a viewpoint of plant pathology, ecology, control, quarantine and legislation, this was very unsatisfactory. Therefore Dye et al. (1975, 1980) proposed to retain the nomenspecies as pathogenic varieties (pathovars, pv.), most of them included in the taxospecies *P. syringae*. The work of e.g. Lelliot et al. (1966), Misaghi & Grogan (1969), Palleroni et al. (1973), Pecknold & Grogan (1973), Sands et al. (1972) showed that some of the pathovars could be distinguished not only by pathogenicity but also by biochemical and by DNA-hybridization tests, and that they possibly deserved a higher taxonomic rank.

The taxonomy of one of the pathovars which differed from *P. syringae* in pathogenicity and in other aspects was studied by me in more detail. This pathovar is *P. syringae* pv. *savastanoi* (formerly named *P. savastanoi*), causing excrescences on *Oleaceae* and *Nerium oleander*. In earlier work (Janse, 1981a, b, 1982), it was found that *pv. savastanoi* could be differentiated from other *P. syringae* forms by biochemical tests and pathogenic reactions. Moreover isolates of this pathovar could be classified into three groups differing in host range, pathogenicity and hormone production. On the basis of these results *P. syringae* pv. *savastanoi* was renamed *P. syringae* subsp. *savastanoi* and three pathovars distinguished, viz. pv. *oleae*, pv. *fraxini* and pv. *nerii*. Results are described in detail in chapter II.1.

The conventional physiological, biochemical and pathogenicity tests which are used in the taxonomy of pseudomonads, present several problems. These tests are generally laborious, time consuming and liable to error due to variation of the bacteria and standardization problems. Discoveries in molecular biology have led to the development of so-called finger-print methods. These methods use genetic material or structural elements such as proteins, cell wall carbohydrates, lipids or fatty acids as a basis for classification and identification (Goodfellow & Minnekin, 1985). Fatty acids have been shown to be useful in classification and identification of bacteria since the sixties (Lechevalier & Lechevalier, 1989). Fatty acid patterns appear to be specific and reproducable, also at low taxonomic level, especially since an automated system

(Microbial Identification System, MIS) has been developed (Miller & Berger, 1985). The MIS and the use of fatty acid analysis in taxonomy is outlined in chapter I.3. Fatty acid analysis has been used in *Pseudomonas* taxonomy, but only to a limited extent (Ikemoto et al., 1978; Oyaizu & Komagata, 1983). I used fatty acid analysis (FAA) in order 1) to try to clarify the taxonomy of several phytopathogenic *Pseudomonas* species and related non-pathogenic species and 2) to investigate if FAA could distinguish between pathogenic varieties so that possibly laborious and time consuming host tests could be circumvented.

In the first place FAA was used in this study to distinguish between the different pathovars of *P. syringae* subsp. *savastanoi*. This bacterium was chosen, because it was well known in our laboratory. It appeared to be possible to separate by FAA the three pathovars from each other and *P. syringae* subsp. *savastanoi* from some other forms of *P. syringae*. Results are dealt with in detail in chapter II.2.

Secondly, an attempt was made to differentiate by FAA pathogenic varieties of *P. solanacearum*, an important non-fluorescent plant pathogenic bacterium which causes a noxious vascular disease 'bacterial wilt' of potato, tomato and many other plants (Bradbury, 1986; Kelman, 1953). Bacterial wilt is in many cases the limiting factor in subtropical and tropical areas. Pathogenic varieties (called races) were described by Buddenhagen et al. (1962) on the basis of pathogenicity tests and colony morphology, viz. race 1 - broad host range, high growth temperature optimum, mainly occurring in tropical areas; race 2 - pathogenic to triploid bananas and related *Heliconia* spp. only; race 3 - strains pathogenic to tomato and potato only, with lower temperature optimum. *P. solanacearum*, especially race 3 may be a threat in temperate areas (Olsson, 1976, Lelliot, 1964) and it received a quarantine status for the Netherlands. A reliable, rapid and clear identification and distinction between pathogenic varieties is therefore important. Discrimination of pathogenic varieties appeared to be possible with FAA. Furthermore I found that *P. solanacearum* can be clearly differentiated from related species in rRNA group II and a similar taxonomic pattern as found in DNA-DNA hybridization studies of Palleroni et al. (1973) was obtained. Results of the study on *P. solanacearum* are presented in chapter II.3.

In the third place the complex of fluorescent *Pseudomonas* bacteria causing soft rot of plants was studied. The soft rot pseudomonads are opportunistic plant pathogens which are very similar to saprophytic pseudomonads belonging to *P. fluorescens* and closely related species, such as *P. aureofaciens*, *P. chlororaphis* and *P. putida*. One group of soft rot strains which was named *P. marginalis* was found to be biochemically indistinguishable from *P. fluorescens* biovar 2 by Stanier et al. (1966).

Because in later studies (e.g. Sands & Hankin, 1975; Wang & Kelman, 1987) soft rot strains were observed which were similar or intermediate to other biovars of *P. fluorescens* or different *Pseudomonas* spp., the name *P. marginalis* was used for any oxidase positive, fluorescent soft rot pseudomonad. We tried to clarify the status of the species name *P. marginalis*, studying a large number of saprophytic and soft rot strains by phenotypic and pathogenicity tests and FAA. Furthermore we attempted to verify the possible soft rot activity of another fluorescent pseudomonad, *P. aeruginosa*, an opportunistic pathogen of animal and man. Finally some non-pectolytic, non-soft rot strains of *P. fluorescens* are described, causing bacterial leaf stripe of *Iris* spp. Results of this study are presented in chapter II.4.

A general discussion on the taxonomy of plant pathogenic pseudomonads and the merits of FAA, is given in chapter III.

'Sehr schwer ist es die Arten der Stäbchenbakterien zu unterscheiden, und ich vermuthe, dass die Zahl der Arten grösser ist, als bisher bekannt'.

Ferdinand Cohn

I.2. Taxonomy of the genus Pseudomonas - a historical overview

Rod shaped, motile bacteria, which are now classified as *Pseudomonas* species are very commonly present in many different habitats and have been observed for a long time. The first systematic study of these and other bacteria was by G.C. Ehrenberg (1838). He created four bacterial genera and one of them, *Bacterium*, contained rod shaped motile and non-motile bacteria. One species, *Bacterium triloculare*, was drawn by him as having a polar flagellar tuft. The optical systems of his time, however, did not allow adequate detection of bacterial flagella. Spherical bacteria were placed in the genus *Monas*, which also included small flagellates belonging to e.g. the gold algae (Family *Chrysophycae)* and zoospores of water fungi. F. Cohn (1872) reserved *Bacterium* for motile, non-chain forming rods and was the first to try to use other than morphological traits for classification, such as pigment formation and pathogenicity. One particular type of organisms associated with blue green abcesses and found to produce the soluble blue green pigment (Schroeter, 1872) was included as *B. aeruginosum*. This bacterium is now known as *P. aeruginosa* and it is the type species of the genus *Pseudomonas*. Cohn still had the opinion that detection of bacterial flagella would bring the flagellated bacteria together with the algae and zoospores belonging to *Monas*. When at the end of the 19th century better optics, such as oil immersion objectives were developed and bacterial flagella were demonstrated by special staining methods, the increase of recognizable morphological details stimulated further classification.

Migula (1894) discriminated between non-motile rods, which he placed in *Bacterium* and motile rods with different flagellar arrangement. Rods with polar flagella (Fig. 1.) were placed in a new genus, viz. *Pseudomonas* and those with peritrichous flagella in *Bacillus*. Migula certainly knew Cohn's ideas about bacteria and the algae and zoospores belonging to *Monas*. Most likely he has choosen the name *Pseudomonas* to indicate that rod shaped, motile bacteria were not similar to the organisms of the genus *Monas*, 'Pseudo' would then mean 'not true or feigned' (from Gr. adj. pseudes = false) and 'monas', a member of the genus Monas (from Gr. monas or monados = unit or monad). The explanation of the name given in Bergey's Manual

(Palleroni, 1984) as 'false monad' is unclear, since the term monad may be used for 'idea' in the sense of Plato, for element or atom in the sense of Leibnitz or for infusoria, in- or excluding bacteria.

Migula's morphological classification of rod-shaped, motile bacteria with polar flagella in *Pseudomonas* was not universally adopted and these bacteria remained to be classified as *Bacterium* or *Bacillus* by others for another 30 years. Also Erwin Smith (1905), the pioneer on plant pathogenic bacteria rejected the use of the name *Pseudomonas*. Up to 1940 plant pathogens with polar flagella can be found to be named as *Bacterium*, *Bacillus*, *Pseudomonas* (including bacteria with 1 polar flagellum, producing yellow non-soluble pigments, now placed in the genus *Xanthomonas*) and *Phytomonas*. In the latter genus which was created by Bergey et al. (1923), plant pathogenicity was the overriding criterion. It also included non-motile species. The work of Dowson (1939, 1943) led to the abandonment of *Phytomonas*, acceptance of *Pseudomonas* for polar flagellated species excluding the yellow plant pathogens, which were placed in the genus *Xanthomonas*.

In the meantime, phenotypic physiological and biochemical determinations had become more and more important in bacteriological classification. This led to a new definition of the genus *Pseudomonas* and the exclusion of many rods showing polar flagella, which, however, were still placed in the family *Pseudomonaceae*. Especially the fluorescent *Pseudomonas* bacteria appeared to be variable and difficult to distinguish by phenotypic tests, leading some investigators to the conclusion that no separation into different species was possible (eg. Rhodes (1959), who placed all the fluorescent forms, including plant pathogens, in one species, *P. fluorescens*). For others, like Krasil'nikov (1959) it was a reason to describe more than 200 species. Also many plant pathogenic fluorescent (nomen) species, most of them oxidase-negative, were described, mainly on the basis of pathogenicity to a particular host or symptoms evoked. In Bergey's Manual of 1957 (Breed et al., 1957) we find the following genus definition:
'Cells monotrichous, lophotrichous or non-motile. Gram-negative. Frequently develop fluorescent, diffusible pigments of a greenish, bluish, violet, lilac, rose, yellow or other color. Sometimes the pigments are bright red or yellow and non-diffusible; there are many species that fail to develop any pigmentation. The majority of the species oxidize glucose to gluconic acid, 2-ketogluconic acid or other intermediates. Usually inactive in the oxidation of lactose. Nitrates are frequently reduced either to nitrites, ammonia or to free nitrogen. Some species split fat and/or attack hydrocarbons. Many species are found in soil and water, including sea water or even heavy brines. Many are plant pathogens; very few are animal pathogens'. *Pseudomonas* contains 148

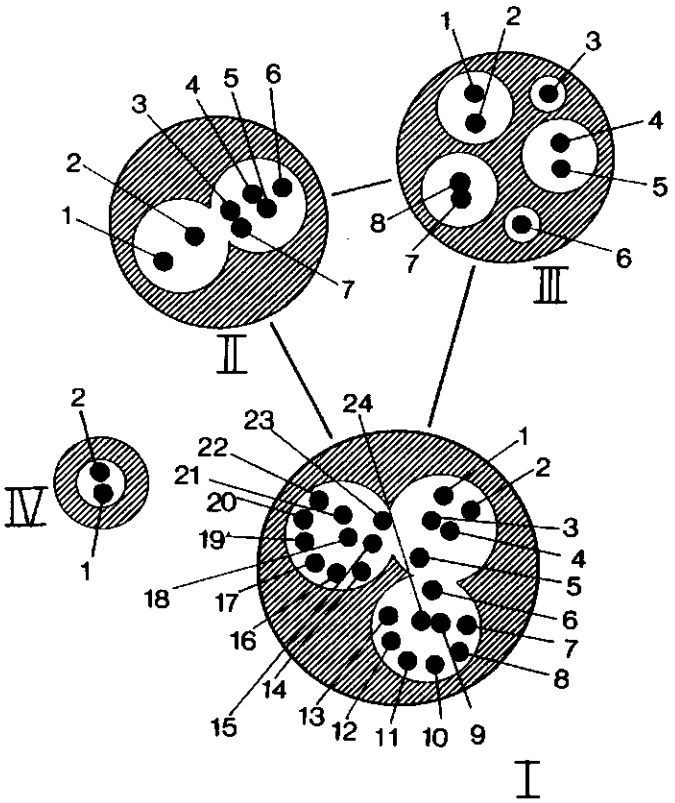

Fig. 2. Arrangement of *Pseudomonas* species, biovars and pathovars according to rRNA and DNA homologies. rRNA homology groups are presented by large circles, DNA homology groups by white circles. Modified from Palleroni (1984). Placement of *P. agarici* and *P. asplenii* in rRNA group I tentative.

rRNA group I: 1, *P. stutzeri*; 2, *P. alcaligenes*; 3, *P. mendocina*; 4, *P. pseudoalcaligenes*; 5, *P. aeruginosa*; 6, *P. putida* bv. A; 7, *P. fluorescens* bv. 5; 8, *P. fluorescens* bv. 4; 9, *P. aureofaciens*; 10, *P. fluorescens* bv. 1; 11, *P. fluorescens* bv. 2; 12, *P. fluorescens* bv. 3; 13, *P. putida* bv. B; 14, *P. asplenii*; 15, *P. syringae* pv. *phaseolicola*; 16, *P.s.* pv. *glycinea*; 17, *P.s.* pv. *tomato*; 18, *P. viridiflava*; 19, *P. syringae* pv. *mori*; 20, *P.s.* pv. *syringae;* 21, *P.s.* subsp. *savastanoi*; 22, *P. agarici*; 23, *P. cichorii*; 24, *P. chlororaphis*.

rRNA group II: 1, *P. solanacearum*; 2, *P. pickettii*; 3, *P. gladioli*; 4, *P. pseudomallei*; 5, *P. mallei*; 6, *P. caryophylli*; 7, *P. cepacia*.

rRNA group III: 1, *P. acidivorans*; 2, *P. testosteroni*; 3, *P. palleroni*; 4, *P. flava*; 5, *P. pseudoflava*; 6, *P. saccharophila*; 7, *P. delafieldii*; 8, *P. facilis*.

rRNA group IV: 1, *P. vesicularis*; 2, *P. diminuta*.

species, 89 of them are plant pathogens. Apart from *Pseudomonas* the genera *Xanthomonas, Acetobacter, Aeromonas, Photobacterium, Azotomonas, Zymomonas, Protaminobacter, Alginomonas, Mycoplana, Zoogloea* and *Halobacterium* are included in the family of the *Pseudomonadaceae*.

The extensive phenotypic studies of (green fluorescent) *Pseudomonas* bacteria by Lysenko (1961), Jessen (1965), Stanier et al. (1966) and Palleroni & Doudoroff (1972) led to a better characterization of (fluorescent) species and a differentiation into biovars of *P. fluorescens*. These authors did consider plant pathogens only to a minor extent. The phenotypic studies of Misaghi & Grogan (1969) and Sands et al. (1970) showed that fluorescent plant pathogens differ substantially from *P. fluorescens* in being much less versatile in attacking carbon compounds. They also showed that several oxidase-negative plant pathogenic *Pseudomonas* nomenspecies did possibly deserve a species rank, while others did not, as they were phenotypically very similar to *P. syringae*. The differentiation within *P. fluorescens* and the difference between this species and the plant pathogens was confirmed by DNA-DNA hybridization studies of Palleroni et al. (1973) and Pecknold & Grogan (1973). Because of the difficulty in discriminating between many fluorescent plant pathogens, using biochemical phenotypic tests, Doudoroff & Palleroni (1974) in Bergey's Manual of Determinative Bacteriology only distinguished *P. syringae, P. cichorii* and *P. fluorescens* (some strains of biovar 2, named *P. marginalis*) as valid plant pathogenic, fluorescent species. The pathogenic individuality of the nomenspecies, however, was completely lost. Therefore Dye et al. (1975, 1980) proposed to retain the nomenspecies as pathogenic varieties (pathovars, pv.), most of them included in the taxospecies *P. syringae*.

The DNA-r(ibosomal)RNA hybridization work of Palleroni et al. (1973), studies on enzymes (Byng et al. 1980; Whitaker et al. 1981a, b) and fatty acid analysis (Oyaizu & Komagata, 1983) have formed and confirmed the most recent taxonomic scheme of the genus Pseudomonas (Fig. 2) as presented in Bergey's Manual of Systematic Bacteriology in 1984 (Palleroni, 1984). Based on the above mentioned and other investigations, the family *Pseudomonaceae* has now only four genera, namely *Pseudomonas, Xanthomonas, Frateuria* and *Zoogloea*. The genus definition for *Pseudomonas* is as follows: 'Straight or slightly curved rods, but not helical, 0.5-1.0 μm in diameter by 1.5-5.0 μm in length. Many species accumulate poly-β-hydroxybutyrate as carbon reserve material, which appears as sudanophilic inclusions. Do not produce prothecae and are not surrounded by sheaths. No resting stages are known. Gram-negative. Motile by one or several polar flagella; rarely non-motile. In some species lateral flagella of shorter wavelength may also be formed.

Aerobic, having a strictly respiratory type of metabolism with oxygen as terminal electron acceptor; in some cases nitrate can be used as an alternate electron acceptor allowing growth to occur anaerobically. Xanthomonadins are not produced. Most, if not all species fail to grow under acid conditions (pH 4.5). Most species do not require organic growth factors. Oxidase positive or -negative. Catalase positive. Chemoorganotrophic; some species are chemolithotrophs, able to use H_2 or CO as energy sources. Widely distributed in nature. Some species are pathogenic for humans, animals or plants. The mol% G+C of the DNA is 58-70 (Bd)'. The genus comprises 27 well described and compared species belonging to four rRNA homology groups (excluding *P. maltophilia,* which is now known to be a *Xanthomonas* species, see Swings et al., 1983), and 50 species with a variable degree of characterization and a largely unknown relation to the well established species. Furthermore 40 pathovars of the oxidase negative, fluorescent plant pathogen *P. syringae* are listed. Confirmation and some extension of this classification has been obtained in recent numerical taxonomic and nucleic acid studies without any fundamental change (Johnson & Palleroni, 1989; De Vos & De Ley, 1983; De Vos et al., 1985; Champion et al., 1980; Woese et al., 1984; Sneath et al., 1981). Some of these authors claim that only rRNA group I should retain the genus name *Pseudomonas,* while the others on the basis of a large rRNA dissimilarity should be regarded as different genera.

A final taxonomy of the genus *Pseudomonas*, however, is still far, if it ever can be reached, which is due to the following problems:

- Conflicting data or conclusions obtained from different determinative techniques.
- Differences in opinion of what should constitute a species of *Pseudomonas*.
- Difficulty in evaluating a larger number of strains by nucleic acid techniques, leading to generalizations based on only a few strains per taxon.
- Difficulty in evaluating and estimating the value of (plant) pathogenicity in taxonomy. Is it just one phenotypic feature or does it reflect a fundamental difference in genetic and/or phenotypic make-up of organisms, or can it be both (also see chapter II).
- Finding of unknown *Pseudomonas* bacteria in habitats which have not been or have only superficially been explored and which behave as intermediates of described forms or as new forms. Especially the fluorescent *Pseudomonas* bacteria are a very complex and ubiquitous group. Most probably only a fraction of the *Pseudomonas* bacteria has ever been described (also see chapter II.4 of this study).

Thus the concluding statements of Palleroni (1978) concerning the complex fluorescent *Pseudomonas* bacteria, namely: 'Unquestionably, many problems remain

unsolved in the taxonomy of the fluorescent organisms. Outside of *P. aeruginosa*, the taxonomic position of many strains of *P. fluorescens* and *P. putida* remains for the moment largely unsolved' probably hold true for the whole genus. This should not be understood as a drawback but as a challenge to study this interesting group of organisms from as many angles as possible.

I.3. Fatty acid analysis as a chemotaxonomic tool

Morphological patterns, which are very important in the classification of most organisms, have shown to be of limited value only for bacteria. Moreover bacterial anatomy can only be revealed by laborious methods in electron microscopy. The discoveries of Louis Pasteur, Robert Koch and others led to the notion that bacteria are responsible for different processes such as the acidification of wine, fermentation of butanol, stimulation of antibody production and anthrax of sheep. From that time physiological, biochemical, serological and pathogenic reactions, determined with pure cultures, were used to create recognition patterns for bacteria. These patterns were used for classification and identification, and a condensation of them in the form of tables and keys over a period of 70 years can be found in Bergey's Manuals of Determinative and Systematic Bacteriology. A definition for such man-made patterns is 'the design made by the relations among a set of objects' (Romesburg, 1984). The contrast between the relations determines the information content of the pattern. The above mentioned conventional patterns are subject to several disadvantages: their generation is usually laborious, expensive and time consuming, their interpretation is liable to errors due to mutations, loss of pathogenicity, choice and quality of test media, etc.

The developments in molecular biology of the past 20 years have generated a lot of knowledge about structure and function of microorganisms, especially of bacteria. It was also discovered that structural elements can be arranged in patterns to be used for discrimination between organisms. Subsequently several chemotaxonomic 'finger-print' methods have been developed, using proteins, nucleic acids, sugars, isoprenoid quinones, mureins, lipids and fatty acids (Goodfellow & Minnekin, 1985). For the 'finger-print' patterns the definition given before also holds true. Interpretation of these patterns is by direct (visual) comparison involving intuition and an expert, by computerized numerical analysis or a combination of both. The finger-print method utilizing whole cell fatty acid analysis, which was used in my studies, will be explained in greater detail.

Principle of fatty acid analysis

For about 30 years (Abel et al., 1963) it has been known that the fatty acid composition of micro-organisms, both qualitative and quantitative, yields a characteristic which is useful for discrimination. It also appeared to be a stable characteristic as the information for fatty acid formation does not occur on plasmids (Van den Boom & Cronan, 1989). More than 300 fatty acids and related compounds such as aldehydes and dimethyl-acetals, have been characterized, most of them also by mass-spectrometry, giving an enormous discriminative potential (Sasser, 1990). An average of cellular fatty acids can be most easily and reproducible obtained by analysis in gas-liquid chromatography, GLC (Horning et al., 1964; Moss, 1981). This average is called here 'fatty acid profile' and it should be distinguished from 'fatty acid pattern', the latter being only a part of the profile. Especially the development of fused silica capillary columns enabled an accurate analysis (Moss et al., 1980). With these columns hydroxy fatty acids and many isomers can be reliably separated. Providing that growth conditions, physiological age of the cells and analysis are well standardized, fatty acid profiles are very reproducible. A high degree of standardization has been realized with an automated system, viz. the Microbial Identification System (MIS) from Microbial ID, Newark, Delaware, USA (Miller, 1982; Sasser, 1990). The MIS has been used in all my studies on bacterial fatty acids.

Fatty acids of bacteria

Bacteria contain lipids in concentrations of 0.2-50%, usually 5-10% of dry weight. For fatty acid analysis (FAA) the lipids containing esterified fatty acids are important. These are mainly:

- phospholipids, structural elements in the cell membrane
- glycolipids, structural elements in the cell membrane, but less common than phospholipids, abundant in Actinomycetes
- lipid A, the lipid part of lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria
- lipoteichoic acids, cell wall components of Gram-positive bacteria

Bacterial fatty acids contain usually 9-20 carbons in a chain. They may be linear, branched, cyclic, saturated, unsaturated (in most cases mono-unsaturated) or containing 2- or 3-hydroxy-groups. Bacteria have the straight chain fatty acids in common with eukaryotes. The mono-unsaturated forms of these straight chains, however, usually have the double bond located at seven, instead of nine carbons from

the terminal methyl group. Unique bacterial fatty acids are the 3-hydroxy, cyclopropane and branched chain fatty acids. In Gram-negative bacteria the main saturated acids are 16:0, 18:0 and often also 14:0, most important unsaturated fatty acids are 16:1 cis or trans 9 and 18:1 cis or trans 11 and hydroxy acids (as part of the LPS) are common. Gram-positive bacteria usually contain important quantities of branched fatty acids (O'Leary, 1967; Ratledge & Wilkinson, 1989a).

The fatty acid content of the lipids and the occurrence of free fatty acids in the bacterial cell may differ qualitatively and/or quantitatively according to the genetic composition and physiological age of the bacterium, nutrients and oxygen available and temperature (Casano et al., 1988, Cullen et al., 1971, Rose, 1988). When grown and harvested under standardized conditions, bacteria show a total, whole cell fatty acid profile which differs from even closely related bacteria.

The use of fatty acid patterns in taxonomy

Fatty acid patterns have shown to be especially valuable for the classification and identification of bacteria (Lechevalier & Lechevalier, 1989). Fundamental and systematic studies on bacteria of many genera have been performed by Moss and colleagues in the USA (Moss, 1981) and by Jantzen and colleagues in Norway (Jantzen et al., 1979). A compilation of fatty acid data of Gram-negative bacteria is given by Ratledge & Wilkinson (1989b). In some cases fatty acid data were compared with those of DNA-DNA and DNA-rRNA homology studies, where a good correlation between these methods was found (Kuykendall et al., 1988; Oyaizu & Komagata, 1983; Sasser & Smith, 1987; this study, chapter II.3). It also appeared to be possible to use FAA for ecophysiological and pathological grouping of bacteria, including plant pathogens. Discrimination was possible below species level (e.g. De Boer & Sasser, 1986; Gitaitis et al., 1987; Janse & Smith, 1990; Mirza et al., 1991; Teurle & Richard, 1987; Urakami & Komagata, 1979; Väisänen & Salkinoja-Salonen, 1989). Especially the development of the fully automated MIS with its powerful pattern recognition program has enabled a proper use of fatty acid patterns for taxonomic purposes. Its components and its use to generate patterns is explained below.

The Microbial Identification System (MIS)

The MIS consists of the following components:

A protocol for standardized growing and harvesting of cells and sample treatment

Hardware from Hewlett Packard

- HP 5180 gas chromatograph with flame-ionisation detector
- integrator, computer with printer, hard disk and disk drive

Calibration mixture of known fatty acids

Software for:

- identification of individual fatty acids
- generation of fatty acid patterns
- data storage
- identification of bacteria
- generation of 'mean' fatty acid patterns (library entries) to build the library (database)
- generation of the database
- statistical programs (cluster analysis and principal component analysis) for taxonomic and epidemiological/ecological studies.

Pattern generation, using the MIS

To obtain a representative average, qualitative and quantitative, of fatty acids present in bacteria belonging to a wide range of genera, the MIS uses the following procedure (Moss et al., 1974; Sasser, 1990):

- Growth of a pure culture of the bacterium for 24 h or 48 h (slow growers) on Trypticase Soy Broth Agar (TSBA) at 28°C. For clinical and anaerobic bacteria different media may be used. The TSBA medium has been chosen because it does not contain fats or fatty acids (Brian & Gardner, 1967).
- Harvest of c.40 mg wet weight of cells from a quadrant of the agar plate showing still confluent growth. Cells in this area are in the late log phase of growth, having a stable fatty acid composition.
- The 40 mg cells are placed in a culture tube and the following steps all take place in this tube: a) saponification for 30 minutes at 100°C in a NaOH/MeOH/water mixture. In this step cells are lysed and fatty acids liberated from lipids; b)

methylation of fatty acids for 10 minutes at 80°C using a MeOH/6N HCl mixture. Methylation renders the fatty acids more volatile in the partially polar column; c) extraction of methyl esters for 10 minutes at room temperature in a hexane/ether mixture; d) a base wash for 5 minutes at room temperature of the extracted methyl esters with dilute NaOH to remove residual acid, reducing contamination of the column (Miller, 1982). The extract is placed in a chromatography vial in the autosampler of the chromatograph and the rest of the process is automatic.

- Chromatographic separation on a 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column, first using a calibration mixture and then samples.
- Calibration to account for drift of apparatus and environmental differences between laboratories. Data from this analysis are used to update the so called 'peak naming table'. This table contains information for the interpretation of raw chromatographic data and identification of (fatty acid) peaks.
- Generation of the fatty acid profile is by identification of peaks by using the updated peak naming table, and an 'elastic' algorithm, where large changes in absolute retention time are allowed, as long as positions between them remain the same. Peak identification is achieved by calculating 'Equivalent Chain Length' (ECL). The ECL can be derived as a function of the retention time of a fatty acid in relation to the retention times of a known series of straight chain saturated fatty acids (Miwa, 1963). The GC and column used allow windows of a peak of only 0.010 ECL, by which great precision in resolution of isomers is achieved.
- The fatty acid pattern is generated by cleaning and adapting the fatty acid profile. Disturbing peaks, which cannot be named, because they do not occur in the peak naming table are not used. These peaks may be caused by electronic disturbances or contaminations. Furthermore, peaks with an almost similar retention time, which are difficult to discriminate, are joined into summed groups. Summed groups may also contain fatty acids which form a more stable character together. An example is 14:0 3OH and its decomposition product 12:0 aldehyde. The sum of both remains the same, while the relative amounts may vary.

Subsequently the fatty acid patterns can be used for identification of the bacterium, library generation and taxonomic, epidemiological or ecological studies.

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II. TAXONOMY OF SELECTED PHYTOPATHOGENIC *PSEUDOMONAS* BACTERIA, USING CONVENTIONAL PHENOTYPIC TESTS AND FATTY ACID ANALYSIS

II.1 *PSEUDOMONAS SYRINGAE* SUBSP. *SAVASTANOI* (EX SMITH) SUBSP. NOV., NOM. REV., THE BACTERIUM CAUSING EXCRESCENCES ON *OLEACEAE* AND *NERIUM OLEANDER* L.

J.D. Janse

International Journal of Systematic Bacteriology 32, 166-169 (1982).

Summary

From a study of the so-called bacterial canker of ash, caused by a variant of "*Pseudomonas savastanoi*" (Smith) Stevens, it became evident that this variant and the variants of "*P. savastanoi*" which cause olive knot and oleander knot can be distinguished from one another on the basis of their pathogenicity and host range. All isolates of "*P. savastanoi*" were recently classified by Dye et al. (Plant Pathol. **59**: 153-168, 1980) as members of a single pathovar of *P. syringae* van Hall. It appears, however, that these isolates differ sufficiently from the other members of *P. syringae* to justify subspecies rank for them. The following classification and nomenclature are therefore proposed: *Pseudomonas syringae* subsp. *savastanoi* (ex Smith) subsp. nov., nom. rev., to include the olive pathogen (pathovar *oleae*), the ash pathogen (pathovar *fraxini*), and the oleander pathogen (pathovar *nerii*). The type strain of *P. syringae* subsp. *savastanoi* is ATCC 13522 (= NCPPB 639).

Introduction

"*Pseudomonas savastanoi*" (Smith 1908) Stevens 1913 was previously used as the name of the bacterium which causes pernicious excrescences on several species of the *Oleaceae*. (Names in quotation marks are not on the Approved Lists of Bacterial Names (Skerman et al.,1980), have not been validly published since 1 Januari 1980, and therefore do not have standing in bacterial nomenclature.) This bacterium was first adequately described from *Olea europea* L. by Smith (1908), who named it "*Bacterium savastanoi*". A variant from the common ash (*Fraxinus excelsior* L.) was then described by Brown in 1932 under the name "*B. savastanoi* var. *fraxini*"; it was later named "*P. savastanoi* subsp. *fraxini*" by Dowson in 1943. The variant from *Nerium oleander* L. (*Apocynaceae*), which was first described by Ferraris in 1926 as

"*P. tonelliana*," was described more adequately by Smith, who renamed it "*P. savastanoi* subsp. *nerii*" in 1928.

My recent studies of the so-called bacterial canker of common ash (Janse, 1981, 1981a) have yielded biochemical, serological, and pathological data that cast additional light on the nature of the isolates obtained from ash, olive, oleander, privet, and jasmin.

In their proposed nomenclature of the plant-pathogenic fluorescent pseudomonads, Young et al. (1978) did not distinguish among the different variants, and they classified all isolates of "*P. savastanoi*" as members of *P. syringae* pathovar *savastanoi*. Their interpretation of the term pathovar, however, is in this case not in agreement with the definition of the term as recommended in the International Code of Nomenclature of Bacteria (Lapage et al., 1975).

According to the available data, the present classification and nomenclature of the organisms under discussion are inadequate. The purpose of this paper is to rectify this situation.

Materials and methods

Bacterial strains. The strains studied are listed in Table 1.

Methods. Descriptions of the methods employed are reported elsewhere (Janse, 1981a).

TABLE 1. Strains used in this study

Strain[a]	Origin	
	Host	Country
"P. savastanoi"		
PD 109	*F. excelsior* L.	Netherlands
PD 116	*F. excelsior* L.	Netherlands
PD 119	*F. excelsior* L.	Netherlands
PD 120	*F. excelsior* L.	Netherlands
PD 159	*F. excelsior* L.	Netherlands
PD 160	*F. excelsior* L.	Netherlands
PD 161	*F. excelsior* L.	Netherlands
PD 179	*F. excelsior* L.	Netherlands
PD 206	*F. excelsior* L.	Netherlands
NCPPB 1464	*F. excelsior* L.	U.K.
NCPPB 1006	*F. excelsior* L.	U.K.
CNBP 1838	*F. excelsior* L.	France
NCPPB 639	*O. europea* L.	Yugoslavia
NCPPB 2327	*O. europea* L.	Italy
NCPPB 640	*N. oleander* L.	Yugoslavia
PD 181	*N. oleander* L.	Spain
NCPPB 2328	*Ligustrum japonicum* Thbg.	Italy
CNBP 1751	*Jasminum* sp.	Greece
P. syringae		
NCPPB 281	*Syringa vulgaris* L.	U.K.
NCPPB 191	*Persea americana* Mill.	Israel
NCPPB 981	*Populus canadensis* Mnch. 'Eugenei'	U.K.
"P. mors-prunorum"		
NCPPB 560	Host unknown	U.K.
"P. maculicola"		
IPO 154	*Brassica olearacea* L.	Netherlands
Saprophytic fluorescent pseudomonads		
PD 117	*F. excelsior* L.	Netherlands
NCPPB 1465[b]	*F. excelsior* L.	U.K.

[a] The strains are maintained under these names in the collections referred to as follows: PD, Culture Collection of the Plant Protection Service, Wageningen, The Netherlands; NCPPB, National Collection of Plant-Pathogenic Bacteria, Harpenden, U.K.; CNPB, Collection Nationale de Bactéries Phytopathogènes, Angers, France; IPO, Culture Collection of the Research Institute for Plant Protection, Wageningen, The Netherlands.

[b] NCPPB 1465 was described as a deviating pathogenic strain of "*P. savastanoi* subsp. *fraxini*" by Šutić and Dowson (1963). In my hands it deviated morphologically, biochemically, and serologically from *P. syringae* subsp. *savastanoi* and was found to be nonpathogenic on four different hosts. It should therefore be regarded as a saprophytic pseudomonad.

Results

Over 40 biochemical and physiological features were determined for the strains. All *"P. savastanoi"* strains had nearly the same characteristics. Significant variances were found only in the production of levan and in the hydrolysis of pectate (not correlated to host plant or pathogenicity) and in the production of indolyl-acetic acid and cytokinin-like compounds. The latter two substances are not produced (or are produced only in very small amounts) by isolates from ash, whereas they are produced by isolates from other host plants. The tests which appear to be useful in differentiating between *"P. savastanoi"* and *P. syringae* are listed in Table 2. For differential characters, also see Sands et al. (1970).

All of the *"P. savastanoi"* strains produced similar titers with an antiserum prepared against strain NCPPB 639 (from *Olea europea*). The antiserum was absorbed with a cross-reacting *P. syringae* strain (NCPPB 191). After cross-absorption, only the *"P. maculicola"* strain showed close antigenic relationship to *"P. savastanoi"* strains.

No significant morphological differences were found between the strains of *"P. savastanoi"*, and only small differences were observed between these strains and the other phytopathogenic pseudomonads tested.

The results of the pathogenicity tests are presented in Table 3. The host plants used were *Fraxinus excelsior* L., *Olea europea* L., *Nerium oleander* L., and *Forsythia intermedia* Zab.

TABLE 2. Biochemical tests useful in differentiating *P. syringae* subsp. *savastanoi* from *P. syringae subsp. syringae*[a]

Tests	*P. syringae* subsp. *savastanoi*	*P. syringae* subsp. *syringae*
Hydrolysis of:		
Gelatin	-	+
Esculin	-	+
Arbutin	- or weak	+
Casein	- or weak	+
Acid from:		
D-(+)-Raffinose	-	+
Erythritol	-	+
Alkali from:		
L-(+)-Tartrate	+	-

[a] Also see Sands et al. (1970).

TABLE 3. Results of pathogenicity tests with isolates of *P. syringae* subsp. *savastanoi* on different host plants.

Isolate(s) from:	Pathogenicity[a] on: Ash	Olive	Oleander	Forsythia
Ash	+	+	-	-
Olive	⊕	⊕	-	-
Oleander	⊕	⊕	⊕	-
Jasmin	-	⊕	-	⊕
Privet	⊕	⊕	-	-

[a] Symbols: +, Necrotic swellings; ⊕, parenchymatous galls; -, no pathogenic reaction

Discussion

From my previous studies (Janse, 1981a) and those of others (Brown, 1932; Sands et al., 1980; Šutić & Dowson, 1963, 1963a; Wilson et al., 1972; Wilson & Magie, 1963), it has become apparent that *"P. savastanoi"* isolates from different hosts are almost indistinguishable morphologically, biochemically, and serologically. Only the production of indolyl-acetic and cytokinin-like compounds differentiated between the isolates from ash and those from other host plants. These substances are most likely related to pathogenicity, as will be discussed below.

However, the isolates from these different host plants show different pathogenicities and host ranges (D'Oleivera, 1939; Janse, 1981a; Smith, 1928; Šutić & Dowson, 1963; Urošević, 1976). The isolates from ash can be clearly distinguished from those of other hosts by a deviating pathogenicity (Šutić & Dowson, 1963; Janse, in press). They evoke wartlike necrotic bark swellings with abundant periderm instead of large, parenchymatous galls. This can possibly be explained by the restricted production of growth substances by isolates from ash. The other isolates of *"P. savastanoi"* were found to produce these substances in rather large amounts in vitro, and elevated levels were also found in their galls (Beltrá, 1961; Surico et al., 1976; Wilson & Magie, 1963). The host range of the isolates from ash is limited to the *Oleaceae*.

The isolates from olive differ from the isolates from ash by producing large galls instead of necrotic bark swellings; they differ from isolates from oleander in host range (Table 3). Isolates from olive usually do not infect *N. oleander*, and it was for this reason that the oleander organism was originally described as a separate species, *"P. tonelliana"* (Ferraris, 1926). Although on two occasions (Pyrowolakis & Welzien, 1974; Wilson & Magie, 1963) strains from olive have been reported to infect *N. oleander*, this is generally not the case.

The isolates from oleander form galls and can therefore be distinguished from isolates from ash; they differ from isolates from olive in host range (Table 3).

On the basis of these facts, it is concluded that the bacterial isolates causing excrescences on ash, olive and oleander must be ranked separately at the level of pathovar as defined in the Bacteriological Code (Lapage et al., 1975) and not as interpreted by Dye et al. (1980). Determination of the status of the jasmin and privet isolates requires a more comprehensive host-range study.

Isolates of *"P.savastanoi"* were recently named *P.syringae* pathovar *savastanoi* (Dye et al., 1980; Young et al., 1978) as a result of investigations (e.g. De Ley, 1968; Misaghi & Grogan, 1969; Sands et al., 1980) which have shown that *"P.savastanoi"* is closely related to *P.syringae* van Hall. However, isolates belonging to *"P.savastanoi"* can be readily distinguished biochemically, serologically, and pathologically from *P.syringae* (Table 2) and its subgroups (Sands et al., 1980); they should therefore be considered at the subspecies level. As the epithet *"savastanoi"* has had no standing in bacterial nomenclature since 1 January 1980 (Skerman et al., 1980), it is here revived for bacterial pathogens causing excrescences on *Oleaceae* and *N. oleander* L.

Description of *Pseudomonas syringae* subsp. *savastanoi* (ex Smith 1908) subsp. nov., nom. rev. (sa.vas.ta'no.i. M.L. gen. noun. *savastanoi* of Savastano, named for L. Savastano, the first to study olive knot).

Gram-negative, nonsporeforming rods with rounded ends, 0.3 to 0.7 by 1.0 to 1.8 μm, occurring singly or in pairs; motile by means of one to five polar flagella. Rather slow-growing, gray-white, smooth, glistening, raised and circular or slightly irregular to undulate colonies are produced on nutrient agar; levan negative or levan positive on nutrient-sucrose (5%) agar; produces a weak, blue-green fluorescent, diffusible pigment on King's B medium; some strains produce a brown diffusible pigment. Metabolism is respiratory. Oxidase negative, catalase positive. Acid is produced from D-(+)-galactose, glucose, D-(+)-ribose, sucrose (slow), D-(+)-xylose, and mannitol; no acid is produced from maltose, D-(+)-raffinose, erythritol, or salicin; alkali is produced from L-(+)-tartrate; esculin, arginine, gelatin, and starch are not hydrolized; generally, arbutin and casein are not hydrolized. Nitrates are not reduced. H_2S is not produced from cysteine. No growth occurs in nutrient broth at 37°C or with 5% NaCl. Hypersensitivity is produced in tobacco leaves. The deoxyribonucleic acid contains 60 mol% guanine plus cytosine (De Ley, 1968). Causes galls and wartlike excrescences on various species of *Oleaceae* and *N. oleander* L. Gall-forming isolates produce indolyl-acetic acid and cytokinin-like substances in vitro.

The type strain of this subspecies is ATCC 13522 (= NCPPB 639). This strain was isolated by D. Šutić from *Olea europea* in Yugoslavia. Its description is identical to that of the subspecies, but with the following modifications: levan negative on nutrient-sucrose (5%) agar; casein hydrolysis is weak; produces galls on *O. europea* L. and *F. excelsior* L.

The following pathovars of *P. syringae* subsp. *savastanoi* are recognized; pathovar *oleae*, causing parenchymatic galls on various species of the *Oleaceae* (Smith, 1908; Stevens, 1913) pathovar *nerii*, causing parenchymatic galls or wartlike excrescences on *N. oleander* L. and various species of the *Oleaeae* (Smith, 1928); and pathovar *fraxini*, causing wartlike excrescences on *F. excelsior* L. and *O. europea* L. (Brown, 1932; Dowson, 1943; Škorić, 1938).

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II.2 PATHOVAR DISCRIMINATION WITHIN *PSEUDOMONAS SYRINGAE* SUBSP. *SAVASTANOI* USING WHOLE CELL FATTY ACIDS AND PATHOGENICITY AS CRITERIA

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Summary

Fifty-two strains of the bacterium *Pseudomonas syringae* subsp. *savastanoi* from six different hosts were characterized by physiological, biochemical and pathogenicity tests and whole cell fatty acid analysis (FAA). Six other *Pseudomonas* strains were included for comparison in FAA. All 52 strains formed a very homogeneous group on the basis of physiological and biochemical tests. On the basis of pathogenicity tests on four different hosts, strains could be divided into the three pathovars of *P. syringae* subsp. *savastanoi* formerly described, viz. pv. *oleae*, pv. *nerii* and pv. *fraxini*. This division into pathovars could be confirmed by FAA, thus showing that FAA is a supportive tool for taxonomic study below species level of plant pathogenic bacteria. Pv. *fraxini* strains (isolated from *Fraxinus excelsior*), formed a discrete cluster in principal component analysis. Pv. nerii strains (isolated from *Nerium oleander*) and pv. *oleae* strains (isolated from *Olea europea*, *Jasminum* sp., *Ligustrum japonicum* or *Phillyrea* sp.) could also be separated but showed close relationship. Two pv. *fraxini* strains showed a deviating (pv. *oleae*) fatty acid pattern. The fatty acids 11:0 iso 3OH, 20:0 and 20:1 trans 11 were found to be exclusively present in pv. *nerii*, but they were not present in all strains. Pathovar discriminative acids were 16:0, 16:1 cis 9, 18:0, 18:1 cis 11 or the ratio of 16:1 cis 9/18:1 or 16:1 cis 9 + 16:0/18:1 + 12:0 2OH.

Introduction

Pseudomonas syringae subsp. *savastanoi* (Smith) Janse is a plant pathogenic bacterium, causing necrotic excrescences or galls on species of the *Oleaceae* and on *Nerium oleander* L. (*Apocynaceae*). On the basis of host range, pathogenesis and plant hormone production three pathogenic varieties (pathovars) of this bacterium have been described (Janse, 1981 a,b, 1982, a,b): pv. *oleae*, causing parenchymatous galls on various species of the *Oleaceae*, pv. *nerii*, evoking parenchymatous galls or wartlike excrescences on *N. oleander* and various species of the *Oleaceae* and pv. *fraxini*,

causing necrotic, wartlike excrescences on *Fraxinus excelsior* L. and to some extent on *Olea europea* L. The first two pathovars produce plant hormones, viz. cytokinins and indole acetic acid, IAA (Evidente et al., 1986; Smidt & Kosuge, 1978). The information for IAA production of pv. *nerii* appears to be located on a plasmid, that of pv. *oleae* on the chromosome (Comai et al., 1982). Pv. *fraxini* does not produce plant hormones (Janse, 1981a).

The discrimination between the pathovars of subsp. *savastanoi*, based on the above mentioned characters, is complicated and/or time consuming. A preliminary study by Varvaro and Sasser (1987) indicated that pv. *oleae* and pv. nerii could be separated by whole cell fatty acids profiles. They did not study strains of pv. *fraxini*, however. Wells et al. (1991), also using whole cell fatty acid analysis could distinguish pv. *fraxini* from the other pathovars but not pv. *oleae* from pv. *nerii*. Fatty acid analysis by gas-liquid chromatography, especially after the development of automated systems (Miller & Berger, 1985) is a rapid and stable method for taxonomic and identification work. Therefore I tried 1) to differentiate a large number of strains of subsp. *savastanoi* into fatty acid groups, eventually correlating with known pathovars, 2) to establish a library for future reference and identification. Some (serologically) related *Pseudomonas* strains were included for comparison.

In this study the classification of 52 strains of subsp. *savastanoi* from six different hosts, using phenotypic biochemical and pathogenicity tests and fatty acid analysis is reported. Furthermore the succesful separation of strains in the three known pathovars on the basis of fatty acid patterns, is demonstrated.

Material and methods

Bacterial strains. Table 4 lists the 58 strains used with their identity and origin. All strains were routinely maintained on nutrient agar (NA, Difco) with 0.1% w/v D-(+)-glucose, and all strains were lyophilized.

Physiological and biochemical properties. Methods used by Janse (1981b) were applied.

Pathogenicity tests. Two-year old seedlings of *Fraxinus excelsior* L., and rooted cuttings of *Olea europea* L., *Nerium oleander* L. and *Forsythia intermedia* Zab. were used. For each isolate 2-4 plants were used and 5 inoculations carried out per plant, inoculating both vigorously growing sprouts and suberized stems during April/May (*F. excelsior* L.) or June/July (other plants). A hypodermic needle and a c. 10^7cells.ml^{-1} suspension in sterile phosphate buffered saline (pH 7.2) of a 24 h NA culture was used for inoculation. After inoculation *F. excelsior* and *F. intermedia* were kept a few days under high humidity (c.20°C, 95% RH) in a glasshouse whereafter

they were grown outdoors. *O. europea* and *N. oleander* were grown in a computer controlled glasshouse at 28°C, 85-90% RH and 10.000 Lux light after inoculation.

Fatty acid analysis. Bacteria were grown for 48 hr at 28°C on Trypticase Soy Broth Agar (TSBA), containing (w/v) 3% Trypticase Soy Broth (BBL) and 1.5% Bacto Agar (Difco). Circa 40 mg (wet weight) cells were harvested from the most dilute quadrant showing confluent growth (late log phase). Whole cell fatty acids were saponified, methylated and extracted, following the method of Miller & Berger (1985). All strains were tested in duplicate.

The Midi Microbial Identification System MIS (Microbiol ID, Inc. Newark, DE, USA) was used. The MIS consists of a Hewlett Packard HP5890A gaschromatograph with a 25 m x 0.2 mm 5% methylphenyl silicone fused silica capillary column, H_2 as carrier gas and a flame-ionization detector, an automatic sampler, an integrator and a computer. The latter identifies the fatty acids, using data of a fatty acid library and a calibration mix of known fatty acids (Microbial ID, Inc.).

Statistical analysis (standard error of differences of the means) of values for some fatty acids or their ratio's was performed using the program GENSTAT on a MicroVAX 3600 computer. Moreover library generating software (LGS) and a statistical program CLUS developed by Microbial ID Inc., were used for principal component and cluster analysis of strains and also for creating a reference library for *P. syringae* subsp. *savastanoi*.

TABLE 4. Origin and identity of strains used in this study

Strain	Host	Country
P. syringae subsp. *savastanoi*		
PD[1]109,116(=PDDCC[2]7711), PD119,120,159 (=PDDCC7712), PD160,161,179,180,206, PD316,392,547,548,555	*Fraxinus excelsior* L.	Netherlands
NCPPB[3]1006 (=PD124)	*Fraxinus excelsior* L.	U.K.
NCPPB1464 (=PD122)	*Fraxinus excelsior* L.	U.K.
CFBP[4]1838 (=PD166)	*Fraxinus excelsior* L.	France
L39-4 (=PD532)	*Fraxinus excelsior* L.	France (L. Gardan)
PD181 (=NCPPB3278),317,390,911,1235	*Nerium oleander* L.	Spain
PD1188,1189,1299,1300	*Nerium oleander* L.	Jordan
L7/12 (=PD529),L14/5 (=PD533)	*Nerium oleander* L.	France (L. Gardan)
NCPPB640 (=PD125)	*Nerium oleander* L.	Yugoslavia
PD1187,1190,1296,1297,1298	*Olea europea* L.	Jordan
PD912,913,914	*Olea europea* L.	Greece

PD1056	*Olea europea* L.	Maroc
PD1265,1266	*Olea europea* L.	Italy (M.Scortichini)
NCPPB639 (=PD118)	*Olea europea* L.	Yugoslavia
NCPPB1481 (=PD 186)	*Olea europea* L.	Yugoslavia
NCPPB2327 (=PD121)	*Olea europea* L.	Italy
T38/1 (=PD534),K23/15 (=PD535)	*Olea europea* L.	France (L. Gardan)
CFBP1751 (=PD167)	*Jasminum* sp.	Greece
T12/4 = PD531	*Jasminum* sp.	France (L. Gardan)
Phil 1 (=PD530), Phil 2 (=PD536)	*Phillyrea* sp.	France (L. Gardan)
NCPPB2328 (=PD185)	*Ligustrum japonicum* Thbg	Italy
P. syringae pv. *syringae*		
NCPPB191 (=PD321)	*Persea americana* Mill.	Israel
NCPPB281 (=PD184)	*Syringa vulgaris* L.	U.K.
P. syringae pv. *morsprunorum*		
NCPPB560 (=PD213)	?	U.K.
P. syringae pv. *maculicola*		
PD236	*Brassica oleracea* L.	Netherlands
Pseudomonas species		
PD117	*Fraxinus excelsior* L.	Netherlands
NCPPB1465 (=PD123)	*Fraxinus excelsior* L.	U.K.

[1] PD, culture collection Plant Protection Service, Wageningen, the Netherlands
[2] PDDCC, Plant Disease Division Culture Collection, Auckland, New Zealand
[3] NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.
[4] CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France

Results

Physiologiocal and biochemical tests

All strains (PD 117, 123, 184, 213, 236, 321 not included, for their deviations from the pattern described below see Janse, 1982a) behaved similarly in physiological and biochemical tests. They were Gram-negative, motile, non-spore forming rods, showing slow growing gray-white, smooth, glistening, raised and circular or slightly irregular to undulate colonies on NA. Usually levan-negative on nutrient-sucrose (5% w/v) agar (PD 120, 161, 179, 180, 206, 555 positive). Strains produced a weak, blue-green fluorescent, diffusible pigment on King's medium B (CFBP 1751, PD179 and PD 534 produced a brown diffusible pigment). Glucose metabolism respiratory, oxidase negative, catalase negative. Acid was produced from D-(+)-galactose, glucose,

D-(+)-ribose, sucrose (slow), D-(+) xylose and mannitol. No acid was produced from maltose, D-(+)-raffinose (PD 161 positive), erythritol or salicin. Alkali was produced from L-(+)-tartrate (PD 120, 392, T12/4 and T38/1 negative). Aesculin and casein were not or only weakly hydrolyzed, arginine, gelatin, starch and arbutin were not hydrolyzed. Nitrates were not reduced, nor was H_2S produced from cysteine. No growth at 37°C or with 5% NaCl. Sodium polypectate was usually hydrolized at pH 5.5 (NCPPB 639, 640, 1464, 2327, 2328, 3278 and PD 1056 negative). Hypersensitivity was produced in tobacco (cv. Samson) leaves (NCPPB 640, PD 1265, 1266, 1296, 1300 negative, L7/12, L24/5, NCPPB 1481, T12/4 doubtful).
On the basis of the above mentioned tests all 52 strains were identical to *P. syringae* subsp. *savastanoi*.

Pathogenicity tests

Results of pathogenicity tests on four different hosts are presented in Table 5. At least three distinct groups could be observed, viz. 1) strains from *Oleaceae* other than *F. excelsior*, causing parenchymatous galls on *O. europea* and *F. excelsior* (the strains from *Jasminum* , also causing parenchymatous galls on *F. intermedia* included), 2) strains from *N. oleander* causing parenchymatous galls or wartlike excrescences on *N. oleander*, *O. europea* and *F. excelsior* and 3) strains from *F. excelsior* causing necrotic excrescences on *F. excelsior* and *O. europea*. On the basis of these results strains from *O. europea*, *Jasminum*, *L. japonicum* and *Phillyrea* could be classified as pv. *oleae*, all strains from *F. excelsior* as pv. *fraxini* and all strains from *N. oleander* as pv. *nerii*, confirming earlier results.

TABLE 5. Results of pathogenicity tests with 52 strains of *P. syringae* subsp. *savastanoi* on 4 different hosts

Strains from	Pathogenicity on: *F. excelsior*	*O. europea*	*N. oleander*	*F. intermedia*
F. excelsior	+	+	-	-
O. europea	⊕[1]	⊕	-	-
N. oleander[3]	⊕[2]	⊕	⊕/+	-
Jasminum	-	⊕[4]	-	⊕
Ligustrum	⊕	⊕	-	-
Phillyrea	⊕	⊕	-	-

+, necrotic swellings; ⊕, parenchymatous galls; -, no pathogenic reaction
1 Strain PD1266 and 1298 doubtful; PD1296 negative
2 Strain PD1300 negative
3 Strain NCPPB640 non pathogenic
4 Strain T12/4 doubtful

Fatty acid analysis

Table 6 shows the mean percentages of fatty acids found in the tested strains of *P. syringae* subsp. *savastanoi*. Over 83% consisted of the straight chain saturated and mono-unsaturated fatty acids 12:0 (c. 4.5%), 16:0 (c. 27%), 16:1 cis 9 (c. 31%) 18:1 cis 11 (c. 20%) and 18:0 (c. 1.5%). About 10.5% were the hydroxy fatty acids 10:0 3OH, 12:0 2OH and 12:0 3OH. The cyclic fatty acid 17:0 was found to be present in rather large amounts (c. 4%). The above mentioned fatty acids were found in all strains. The other 14 fatty acids (in total c. 1.5%) occurred infrequently. The fatty acids 15:0 (1 x NCPPB 2327) 17:0, and 19:1 trans 7 have never been reported before to occur in *P. syringae* subsp. *savastanoi*. The fatty acids 11 : 0 iso 30H, 19:0 iso, 20 :1 trans 11 and 20:0 occurred in strains from *N. oleander* only, they were not present in all *Nerium* strains, however. The fatty acid pattern of the other *Pseudomonas* strains differed to a large extent from that of *P. syringae* subsp. *savastanoi* (table 7) resulting in low similarity values (below 0.5) with the *P.s.* subsp. *savastanoi* library.

TABLE 6. Total cellular fatty acid profile of 52 strains* of *Pseudomonas syringae* subsp. *savastanoi* grown for 48 h on TSBA medium

fatty acid	count	mean%	s.d.	fatty acid	count	mean%	s.d.
saturated				hydroxy			
12:0	104	4.5	0.4	10:0 3OH	104	3.1	0.3
14:0	63	0.2	0.2	11:0 iso 3OH	8	t	
15:0	1	t		12:0 2OH	104	3.2	0.2
16:0	104	27.1	3.3	12:0 3OH	104	4.5	0.3
17:0	17	t					
18:0	103	1.5	0.8	unsaturated			
20:0	2	t		16:1 cis 9	104	31.6	3.3
				18:1 cis 11	104	19.8	5.8
branched				19:1 trans 7	5	t	
17:0 iso	44	0.3	0.4	20:1 trans 11	7	t	
19:0 iso	1	t					
				unknown/			
				19:0 cyclo	56	0.4	0.4
cyclo				C 9-10			
17:0 cy	103	3.7	2.05				
19:0 cy							
C11-12	8	t					

- = not detected; t = trace
* all strains tested in duplicate

TABLE 7. Percentage of several fatty acids of some *Pseudomonas* bacteria* related to *P. syringae* subsp. *savastanoi*

Bacterium	Fatty acid				
	12:0 2OH	16:0	16:1cis9	18:0	18:1cis11
P. syringae pv. *syringae* (NCPPB191)	3.7	20.7	36.1	0.5	17.0
P. syringae pv. *syringae* (NCPPB281)	3.7	25.0	34.7	1.2	17.6
P. syringae pv. *morsprunorum* (NCPPB560)	3.5	26.4	30.6	1.5	17.4
P. syringae pv. *maculicola* (PD236)	3.0	25.5	36.9	1.2	18.4
Pseudomonas sp. (PD117)	0	25.7	26.0	0.2	18.7
Pseudomonas sp. (NCPPB1465)	0	19.6	4.9	0.9	32.7
P. syringae subsp. *savastanoi*	3.2	27.1	31.6	1.5	19.8

* result of a test in duplicate

Statistical significant differences between concentrations of fatty acids or their ratios (Table 8) were correlated with the segregation of strains in three groups (pathovars) which was found in the pathogenicity tests. There was greater difference between strains from *F. excelsior* and those from other hosts than between strains from *N. oleander* and those from *Oleaceae* other than *F. excelsior*. Only four deviating strains were found, two strains from *F. excelsior* (NCPPB 1006 and CFBP 1848) repeatedly showed a fatty acid pattern of pv. *oleae*, one strain from *N. oleander* (PD 1300) showed a pattern of pv. *oleae* and one strain from olive (NCPPB 1481) showed a pattern of pv. *nerii*. Principal component analysis showed that pv. *fraxini* formed a separate group, but that pv. *oleae* and *nerii* had an overlap for these features (Fig. 3). This suggests a closer relationship between pv. *oleae* and pv. *nerii* than between pv. *fraxini* and the other two pathovars.

TABLE 8. Fatty acids or their ratios that differentiate pathovars of *P. syringae* subsp. *savastanoi* (P = 0.05)[1]

Fatty acid	pv. *fraxini* mean %	pv. *nerii* mean %	pv. *oleae* mean %	s.e.d.
16:0	31.03	23.85	25.47	0.471
16:1 cis 9	34.81	28.56	30.28	0.634
18:0	0.98	2.31	1.64	0.162
18:1 cis 11	12.69	25.86	22.15	0.755
16:1 cis 9/18:1	2.82	1.16	1.37	0.089
16:1 cis 9+16:0/18:1+12:0 2OH	4.24	1.88	2.21	0.118

[1] Based on data of 50 strains tested in duplicate (deviating strains NCPPB 1006 and CFBP 1838 excluded)

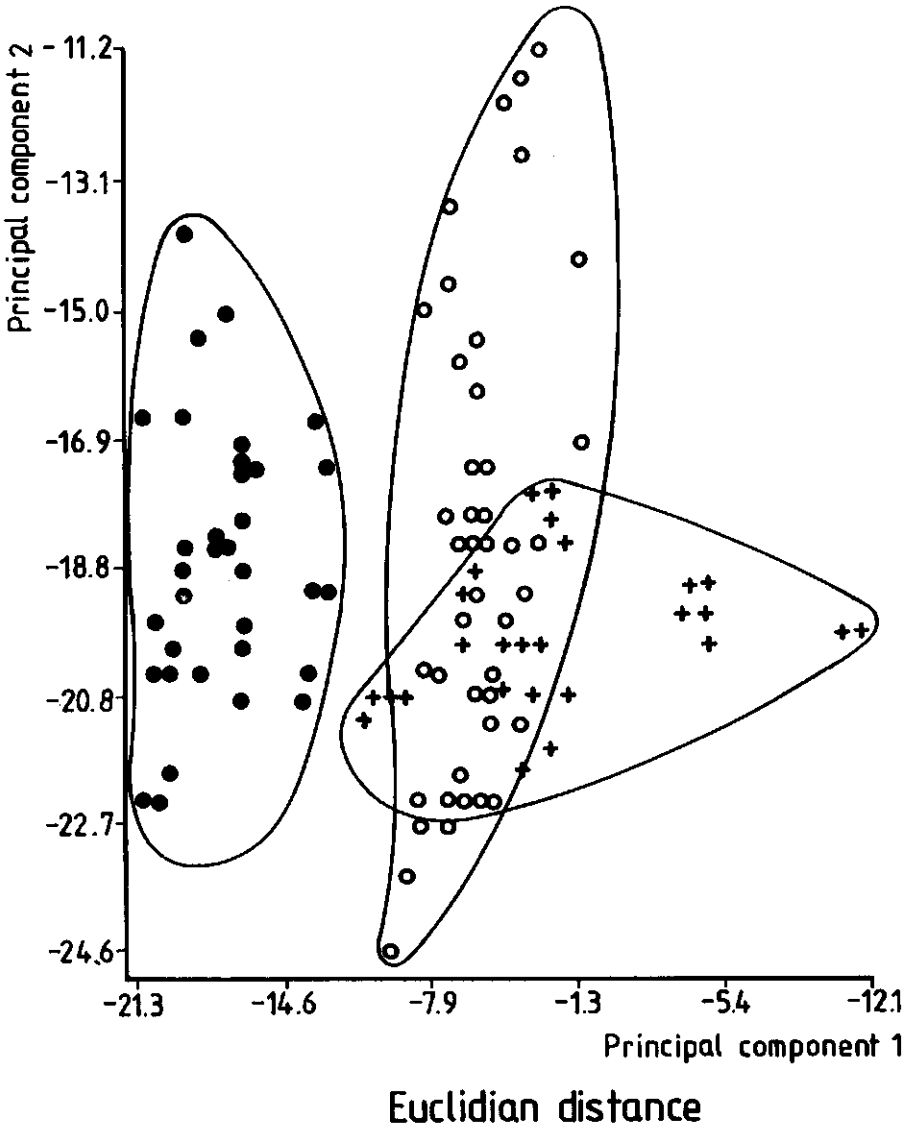

Fig. 3. Two-dimensional plot of principal component analysis of 52 strains of *Pseudomonas syringae* subsp. *savastanoi* (tested in duplo) showing subdivision in three fatty acid groups. These groups are corresponding to pv. *fraxini* (●), pv. *oleae* (0) and pv. *nerii* (+)

This study shows once more that fatty acid analysis can be a supportive tool for classification of plant pathogenic bacteria below species level, as was also reported by De Boer and Sasser (1986) for subspecies of *Erwinia carotovora*. Strains of *P. syringae* subsp. *savastanoi* allocated to pv. *oleae* or pv. *nerii* based on pathogenicity and host range could be separated into two similar groups by fatty acid analysis, confirming results of Varvaro and Sasser (1987). Wells et al. (1991) could not separate pv. *oleae* from pv. *nerii*, possibly because they used a different medium, six-day old cells, a different column and a different chromatograph.

The differences in fatty acid profile of pv. *oleae* and pv. *nerii* correlated well with those found by Varvaro and Sasser (1986), even though they used 24h old cells instead of 48hr old cells. I found, however, that many strains do not produce reproducable amounts of usable cell material after 24h, yielding variable profiles. The acids 11:0 iso 3OH, 20:0 and 20:1 trans 11 were also found by me to be exclusively present in pv. *nerii*. However 17:0 iso was also found to occur after 48h cultivation in pv. *oleae* and to a minor extend in pv. *fraxini*. While strains from *L. japonicum* and *Jasminum* showed some differences in host range as compared to strains from *Olea europea*, these strains all produced parenchymatous galls and showed a fatty acid profile similar to that of olive strains. Varvaro and Sasser (1987) found a similar pattern studying a larger group of strains from *L. japonicum*. Therefore no further subdivision into pathovars was thought to be useful and justified at the moment. The occurrence of new pathovars may not be excluded however since a new host has recently been described, viz. *Myrtus* sp. (Gardan and Abu Ghorrah, 1987). The strains allocated to pv. *fraxini* on the basis of pathogenicity and host range could be clearly separated from those belonging to pv. *nerii* or pv. *oleae*, confirming results of Wells et al. (1991).

Several deviating strains were found, not fitting in the pathovar pattern, as far as their fatty acids were concerned. Two strains from *F. excelsior*, one from France and one from the U.K. showed a pv. *oleae* profile. Other *F. excelsior* strains from these countries showed the pv. *fraxini* pattern. The basis and frequence of this deviation are unknown and need further study, other characters were consistent for pv. *fraxini*. Strains with the pathogenic phenotype of pv. *nerii* have been isolated from olive in the Middle East (Pyrolowakis and Welzien, 1979; Wilson and Magie, 1963). The strain isolated from pv. *nerii* phenotype was from this area, however, it did not show the pathogenic phenotype of pv. *nerii*. This means that deviating strains (i.e. host not correlating with pathovar classification in fatty analysis), can only be definitely identified by a pathogenicity test on different hosts.

This study on fatty acids thus confirms that the existence of the three pathovars previously described (Janse, 1982b) can be confirmed by fatty acid analysis. It must be remarked that pv. *nerii* and pv. *oleae* upon principal component analysis showed a close relationship and a partly overlap. Pv. *fraxini* was much more different. This correlates with the pathogenic properties and geographic distribution of the different pathovars. Pvs *nerii* and *oleae* both produce parenchymatous galls and growth promoting hormones (Surico et al., 1985). They occur mainly in the Middle East/Southern Europe area. Pv. *fraxini* produces necrotic excrescences and no hormones, it occurs mainly in the Middle/Western Europe area. This study also shows that the pathovars of *P. syringae* subsp. *savastanoi* show more differential characters and are therefore more natural (polythetic) than originally suspected. The definition of pathovar used here is that of the Bacteriological Code (Lapage et al., 1975): pathogenic to one or more hosts, and not the one used by Dye et al., (1980) where it has almost the definition of subspecies. When using the former definition, there would be no need for designating type strains of pathovars (pathotypes, as defined by Dye et al. 1980) since the concept of pathovar does not fall under the rules of the Code.

Pathotypes, however, were mentioned by me in an abstract (Janse, 1987) and because the pathovars could be eventually elevated in rank, they are repeated here for valid publication:
strain PD 118 = NCPPB 639 = ATCC 13522 as pathotype for pv. *oleae*
(also type strain for subspecies *savastanoi*)
strain PD 181 = NCPPB 3278 as lectopathotype for pv. *nerii*
strain PD 116 = PDDCC 7711 as pathotype for pv. *fraxini*.

When several (phenotypically or serologically) related *Pseudomonas* strains were compared in fatty acid analysis, *P. syringae* subsp. *savastanoi* could be clearly differentiated from them. This was also true for a saprophytic strain (NCPPB 1465) which was originally described as a deviating pathogenic strain, namely *P. savastanoi* subsp. *fraxini* (Šutić and Dowson, 1963). Moreover a hypersensitivity positive *P. syringae* strain isolated from a lesion on *F. excelsior* with a close though deviating phenotypic profile, serologically cross-reacting, but not pathogenic for ash could be easily differentiated by fatty acid analysis from subsp. *savastanoi* (Janse, unpubl. results). These findings suggest caution when postulating all kind of transitions in the *P. syringae* group especially when studying strains from the phyllosphere and not from active lesions or galls (Ercolani, 1983).

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II.3 INFRA- AND INTRASPECIFIC CLASSIFICATION OF *PSEUDOMONAS SOLANACEARUM* STRAINS, USING WHOLE CELL FATTY ACID ANALYSIS

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Summary

Whole cell fatty acid patterns of 69 strains of the plant pathogenic bacterium *Pseudomonas solanacearum* were determined by capillary gas liquid chromatography (GLC). Strains originated from 17 different hosts from many parts of the world covering four biovars and three (pathogenic) races as well as *Zingiber* strains. Identity of strains was confirmed by conventional biochemical and pathogenicity tests. Multivariate analysis of fatty acid data produced four infraspecific clusters. Three of these clusters corresponded to races 1, 2 and 3, the fourth consisted of five deviating race 1, biovar 1 strains from the U.S.A. Biovar 4 strains of *Zingiber* were found in the race 1 cluster. Only one biovar 3 strain from this host behaved differently. In contrast to conventional tests it was possible to discriminate between avirulent strains of different races. From unknown strains the race can be determined, using a reference library. The use of the term 'pathovar' instead of 'race' is discussed.

Intraspecific relationships between *P. solanacearum* and four related species (*P. caryophylli, P. cepacia, P. gladioli, P. pickettii*) of rRNA group II were studied, also using fatty acid analysis. The taxonomic pattern found in DNA-DNA homology studies of these species was confirmed.

Keywords: GLC - multivariate analysis - avirulent strains - *Pseudomonas caryophylli* - *Pseudomonas cepacia* - *Pseudomonas gladioli pv. alliicola* - *Pseudomonas gladioli pv. gladioli* - *Pseudomonas pickettii.*

Introduction

Pseudomonas solanacearum (Smith) Smith is an important plant pathogenic bacterium, causing a vascular disease, resulting in wilting and death of plants. Early descriptions of this disease on potato and tomato were given by Halsted in 1892 in the U.S.A. (Kelman, 1953) and on tobacco by Janse (1892) in Indonesia. Subsequently it

was described in much detail on tomato, eggplant and potato by Smith (1896) in the U.S.A. Smith also gave a description of the bacterium, naming it *Bacillus solanacearum*. Since that time many host plants, including important food crops, have been discovered in more than 35 families of plants in tropical, subtropical and (warm)temperate regions of the world (Bradbury, 1986; Hayward, 1986).

Already in the early years differences in pathogenicity between strains of *P. solanacearum* were noticed (Buddenhagen & Kelman, 1964), but only 65 years after description of the bacterium a serious attempt for infra-specific subdivision was made by Okabe & Goto (1961). On the basis of detailed studies they distinguished at least 13 pathogenic types from Solanaceous hosts. Buddenhagen et al. (1962) classified several hundred strains on the basis of host range studies and colony morphology into three (pathogenic) races, viz. race 1 - broad host range strains with high growth-temperature optimum; race 2 - strains pathogenic to triploid bananas and related *Heliconia* spp. only; race 3 - strains pathogenic to potato and tomato only, with lower temperature optimum. Later two other races have been described, namely certain strains from *Zingiber officinale* as race 4 (Buddenhagen, 1986) and strains from *Morus alba*, also as race 4 (He et al., 1983). The status of the latter two races is not yet clear, however (Buddenhagen, 1986).

Hayward (1964) discovered variation in biochemical behaviour of *P. solanacearum* strains and described four biovars. These biovars are not correlated with races, except biovar 2, which is linked to race 3. Serology has not been of much help in subdivision or finding correlation with host range (Schaad et al, 1978), though Morton et al (1966) claimed to have obtained race specific antisera. A similar situation as in serology was found in nutritional studies and infraspecific DNA-DNA-hybridizations (Harris, 1972; Okabe & Goto, 1961; Palleroni & Doudoroff, 1971) with one possible exception (Engelbrecht & Hatting, 1989).

Recently restriction fragment length polymorphism (RFLP) analysis, using DNA probes has been found useful to distinguish different (pathogenic) types and to verify the race of *P. solanacearum* strains (Cook & al., 1989). With this method, it is not possible to determine the race of strains with new RFLP patterns. To know whether one deals with a broad host range strain or not, these strains have first to be race-typed by pathogenicity tests.

Fatty acid analysis has been found to be a supportive tool for classification and identification of plant pathogenic bacteria below species level (De Boer & Sasser, 1986; Janse, 1991). Fatty acids of *P. solanacearum* have been studied to a limited extent (Oyaizu & Komagata, 1983), however. If (pathogenic) races could be

distinguished by fatty acid analysis, determination of race could be easily obtained, circumventing laborious host tests. Therefore whole cell fatty acid patterns were determined of 69 strains of *P. solanacearum* from 17 different host plants and from different parts of the world. These patterns were examined for infraspecific subdivision. To be sure of the identity of strains, biochemical and pathogenicity tests were carried out.

Correlation between ribosomal RNA and DNA homologies and fatty acid composition in the genus *Pseudomonas* has been observed (Oyaizu & Komagata, 1983; Sasser & Smith, 1987). In RNA/DNA studies *P. solanacearum* has been placed in the so-called ribosomal RNA group II, together with *P. caryophylli*, *P. cepacia*, *P. gladioli*, *P. pickettii*, *P. mallei* and *P. pseudomallei* (Palleroni et al., 1973). Using the first four of these species and *P. solanacearum* I examined whether relationships found by DNA-DNA hybridizations could be confirmed by fatty acid analysis. This should also give some idea about the genetic basis of infraspecific differences found in fatty acid analysis.

In this article the successful separation of *P. solanacearum* strains into the known three (pathogenic) races, using fatty acid analysis is reported. Furthermore the confirmation by fatty acid analysis of relationships between *P. solanacearum* and related *Pseudomonas* spp. in ribosomal RNA group II as determined by DNA-DNA hybridizations, is described.

Material and methods

Bacterial strains. Table 9a and b list the bacterial strains used with their classification and origin. Some strains were isolated recently, others have been in culture collections for many years. All strains were lyophilized and routinely grown on nutrient agar (NA, Difco). *P. solanacearum* strains were also maintained in sterile distilled water as working stock cultures.

Physiological and biochemical tests. In order to verify identity of *Pseudomonas* spp. received from diverse culture collections a few key tests were performed (Table 12). Methods applied were generally according to Palleroni & Doudoroff (1972). Acid production from carbohydrates was studied in the medium of Hugh & Leifson (1953). For gelatin hydrolysis and arginine dihydrolase the methods of Lelliot et al. (1966) were used. When results were obtained which deviated from the pattern described by Palleroni & Doudoroff (1972), tests were repeated.

Biovar determination. To determine or verify biovars of *P. solanacearum*, strains were grown in the minimal medium of Hayward (1964). In this medium they were

checked for the ability to oxidize three disaccharides (cellobiose, lactose, maltose) and three hexose alcohols (dulcitol, mannitol and sorbitol). Biovar 1 does not oxidize any of these carbon compounds, biovar 2 the disaccharides only, biovar 3 all of these and biovar 4 the hexose alcohols only.

Pathogenicity tests. *Lycopersicum esculentum* 'Moneymaker', *Nicotiana tabacum* 'White Burley', *Solanummelongena* 'Black Beauty' from seed, *Musa acuminata* (syn. *cavendishii*) from cuttings and *Zingiber officinale* from cutted rhizomes were used. Two (ginger, tobacco, banana) or five (eggplant, tomato) plants in the 1st to 3rd true leaf stage were placed in 75 cm-long trays with pasteurized potting soil and grown in a computer controlled, insect free glasshouse. Each *P.solanacearum* strain was inoculated with a 23 G hypodermic needle, using a 10^7 cells.ml^{-1} suspension of a 24-48 h NA culture in sterile physiological buffered saline (PBS, pH 7.2). Bacterial concentration was adjusted using a BaSO4 standard (Kiraly, 1974). Injection was at air/soil level in the stem. With ginger ca.1 ml of bacterial suspension, containing 0.5% v/v Tween 20, was also poured into the leaf whorl. Control plants (four per test plant species) were inoculated as described above, using sterile PBS. After inoculation plants were kept at 28°C ± 2°C, ca.85% RH and 10.000 lux light, using mercury lamps. Observations were performed up to four or eight weeks (banana) after inoculation. From each test plant species reisolations were made which were confirmed by colony morphology on yeast-peptone-glucose agar (YPGA, containing (w/v 0.5% yeast extract, 1% peptone, 0.5% D(+)gucose and 1.5% agar).

Race discrimination by the hypersensitivity reaction (HR) on tobacco 'White Burley' was performed as described by Lozano & Sequira (1970). Bacterial suspension of ca.10^9 cells.ml^{-1}, prepared as described above, were infiltrated into intercostal tissue of at least two leaves. Plants were kept under the same conditions as used for the inoculations and observed after 24 hr and up to three weeks after infiltration.

Fatty acid analysis. Bacteria were grown for 48 h at 28°C on Trypticase Soy Broth Agar (TBSA), containing (w/v) 3% Trypticase Soy Broth (BBL) and 1.5% Bacto-Agar (Difco). Harvesting, gas-chromatographic analysis, reference library generation and principal component and cluster analysis were performed as described by Janse (1991). All strains were tested in duplicate.

TABLE 9a. Origin and identity of *Pseudomonas* strains other than *P. solanacearum*, used in this study

Strain	Received from/ Alternate numbers	Host	Country
P. caryophylli			
PD[1] 977	= NCPPB[2] 2151(T)	*Dianthus caryophyllus*	USA
P. cepacia			
PD 959	= NCPPB 2993 (T)	*Allium cepa*	?
PD 961	= NCPPB 3025	*Allium cepa*	Italy
PD 1285	A.H.Aziz, 4	blood	Malaysia
PD 1286	A.H.Aziz, 5	blood	Malaysia
PD 1312	A.H.Aziz, 6b	blood	Malaysia
PD 1489	= LMG[3] 6995	male patient	Sweden
PD 1490	= LMG 2161	forest soil	Trinidad
PD 1491	= LMG 6859	rotting tree trunk	Trinidad
PD 1492	= LMG 6962	*Allium cepa*	USA
PD 1493	= LMG 6981	bronchial wash	?
PD 1494	A.C. Hayward, UQM[4] 3153	mushroom cap	Australia
PD 1495	A.C. Hayward, UQM 3152	sugarcane soil	Australia
PD 1499	= IBSBF[5] 567 = NCPPB 1962	?	?
PD 1500	= IBSBF 588 = ATCC 17759	forest soil	?
PD 1501	M. Goto, Pcpl 10	?	Japan
PD 1519	P. Cawley, NHI[6] 76/0164	nasal swab	N. Zealand
PD 1520	P. Cawley, NHI 76/1316	urine	N. Zealand
PD 1521	P. Cawley, NHI 77/0120	wound (gastrectomy)	N. Zealand
P. gladioli pv. *alliicola*			
PD 973	= NCPPB 947 (NP)	?	?
PD 1507	= LMG 6955	*Allium cepa*	Australia
PD 1508	= LMG 6957	?	USA
PD 1516	= IBSBF 541	*Allium cepa*	Brazil
PD 1518	= NCPPB 2478	*Allium cepa*	India
P. gladioli pv. *gladioli*			
PD 981	= NCPPB 1891 (PVRS)	*Gladiolus* spec.	?
PD 1502	= NCPPB 1887	*Gladiolus* spec.	USA
PD 1504	= IBSBF 546 = NCPPB 1891 (PRVS)	*Gladiolus* spec.	?
PD 1505	= IBSBF 589	*Asplenium nidus*	USA
PD 1572	= MAFF[8] 03-01588	*Cymbidium* spec.	Japan

TABLE 9a (continued)

Strain	Received from/ Alternate numbers	Host	Country
P. pickettii			
PD 1287	A.H.Aziz 8	eye swab	Malaysia
PD 1288	A.H.Aziz 9	eye swab	Malaysia
PD 1289	A.H.Aziz 10	eye swab	Malaysia
PD 1509	P. Cawley, NHI 74/1928	blood culture	N. Zealand
PD 1510	P. Cawley, NHI 76/0249	waterbath, hospital neonatal unit	N. Zealand
PD 1511	P. Cawley, NHI 76/2402	liquor (pregnant)	N. Zealand
PD 1512	P. Cawley, NHI 76/2641 = NZRM7 1233	blood culture	N. Zealand
PD 1513	P. Cawley, NHI 76/2779	blood culture	N. Zealand
PD 1514	P. Cawley, NHI 76/3915 = NZRM 2020	crystal violet solution	N. Zealand
PD 1515	P. Cawley, NHI 81/0160 (*P. thomasii*)	wound	N. Zealand

legend: see Table 9b

TABLE 9b. Origin and identity of 69 strains of *P. solanacearum* used in this study.

Strain	Received from/ Alternate numbers	Race	Biovar	Host	Country
PD[1] 1255	J.C. Girard, An-1	1	3	*Anthurium x andreanum*	Reunion (FR.)
PD 1424	M. Goto, A6	1	4	*Arachis hypogea*	Japan
PD 1257	J.C. Girard, P1	1	3	*Capsicum frutescens*	Reunion (FR.)
PD 1417	M. Goto, pepper 3	1	4	*Capsicum* spec.	Japan
PD 1454	A. Kelman, UW[9] 195	1	4	*Capsicum* spec.	Philippines
PD 1432	= DAR [10] 34824	1	3	*Cucurbita moschata*	Australia
PD 1422	M. Goto, SB 3-3	1	3	*Fragaria* spec.	Japan
PD 1446	A. Kelman, UW 6	2	1	*Heliconia* spec.	Costa Rica
PD 1453	A. Kelman, UW 170	1	1[a]	*Heliconia* spec.	Columbia
PD 511	= NCPPB[2] 325 = K60-1 (NT)	1	1	*Lycopersicon esculentum*	U.S.A.
PD 1449	A. Kelman, K74 = UW 26	1	1	*Lycopersicon esculentum*	U.S.A. (GA)
PD 1647	R.A. Stall, 9	1	1	*Lycopersicon esculentum*	U.S.A. (FA)
PD 1648	R.A. Stall, K60 = UW 25	1	1	*Lycopersicon esculentum*	U.S.A.. (NC)
PD 1650	R.A. Stall, 2728	1	1	*Lycopersicon esculentum*	U.S.A. (FA)
PD 1651	R.A. Stall, BF1	1	1	*Lycopersicon esculentum*	U.S.A. (FA)
PD 1258	J.C. Girard, T5	1	3	*Lycopersicon esculentum*	Reunion (FR)

TABLE 9b. (continued)

Strain	Received from/ Alternate numbers	Race	Biovar	Host	Country
PD 1434	= DAR 34822	1	3	*Lycopersicon esculentum*	Australia
PD 1437	= DAR 61442	1	3	*Lycopersicon esculentum*	Australia
PD 1438	= DAR 61443	1	3	*Lycopersicon esculentum*	Australia
PD 1573	= MAFF[8] 03-01522	1	3	*Lycopersicon esculentum*	Japan
PD 1646	R.A. Stall, 118	1	3	*Lycopersicon esculentum*	Sri Lanka
PD 1421	M. Goto, T19-2	1	4	*Lycopersicon esculentum*	Japan
PD 1445	A. Kelman, UW3	2	1	*Musa* spec.	Panama
PD 1447	A. Kelman, UW 11	2	1	*Musa* spec.	Costa Rica
PD 1653	R.A. Stall, S 228	2	1	*Musa* spec.	Honduras
PD 1456	A. Kelman, UW 213	1	1	*Nicotiana* spec.	U.S.A.
PD 1420	M. Goto, B1-3	1	3	*Nicotiana* spec.	Japan
PD 1429	H. Hara, Ps 51	1	3	*Nicotiana* spec.	Japan
PD 1436	H. Hara, Ps 28	1	3	*Nicotiana* spec.	Japan
PD 1433	A.C. Hayward UQM 012 BS	1	3	*Rapistrum rugosum*	Australia
PD 1423	M. Goto, G1-1	1	3	*Sesamum indicum*	Japan
PD 1655	R.A. Stall, 1317	1	1	*Solanum melongena*	U.S.A. (FA)
PD 1256	J.C. Girard, Au 22	1	3	*Solanum melongena*	Reunion (FR)
PD 1652	R.A. Stall, 131	1	3	*Solanum melongena*	Sri Lanka
PD 1458	A. Kelman, UW 356, ex He	1	4	*Solanum melongena*	China
PD 1414	= IBSBF[5] 110	1	1	*Solanum tuberosum*	Brasil
PD 1428	A.C. Hayward UQM, 01059	3	2	*Solanum tuberosum*	Australia
PD 426		3	2	*Solanum tuberosum*	Egypt
PD 427		3	2	*Solanum tuberosum*	Egypt
PD 441	K. Olsson	3	2	*Solanum tuberosum*	Sweden
PD 510	= NCPPB 909	3	2	*Solanum tuberosum*	Egypt
PD 1100		3	2	*Solanum tuberosum*	Egypt
PD 1101		3	2	*Solanum tuberosum*	Egypt
PD 1122		3	2	*Solanum tuberosum*	Egypt
PD 1254	G.S. Shekhawat Ps 40	3	2	*Solanum tuberosum*	India
PD 1260	J.C. Girard PdI 7-6	3	2	*Solanum tuberosum*	Reunion (FR)
PD 1261	J.C. Girard PdT 12-4	3	2	*Solanum tuberosum*	Reunion (FR)
PD 1262	M.A. El Goorani	3	2	*Solanum tuberosum*	Egypt
PD 1263	M.A. El Goorani	3	2	*Solanum tuberosum*	Egypt
PD 1408	E. French 269	3	2	*Solanum tuberosum*	Chile
PD 1409	E. French 270	3	2	*Solanum tuberosum*	Peru
PD 1415	= IBSBF 147	3	2	*Solanum tuberosum*	Brasil
PD 1435	= DAR 49321	3	2	*Solanum tuberosum*	Australia
PD 1440	= DAR 41293	3	2	*Solanum tuberosum*	Australia

TABLE 9b. (continued)

Strain	Received from/ Alternate numbers	Race	Biovar	Host	Country
PD 1649	R.A. Stall, 73	3	2	*Solanum tuberosum*	Sri Lanka
PD 278		1	3	*Solanum tuberosum*	Indonesia
PD 1259	J.C. Girard PdT9-5	1	3	*Solanum tuberosum*	Sri Lanka
PD 1654	R.A. Stall, 133	1	3 [b]	*Solanum tuberosum*	Sri Lanka
PD 1410	E. Frenck, 195	1	4	*Solanum tuberosum*	Sri Lanka
PD 1419	M. Goto, P 66	1	4	*Solanum tuberosum*	Japan
PD 1431	E. French, 148	1	4	*Solanum tuberosum*	Philippines
PD 1450	A. Kelman, UW 51	1	4	*Solanum tuberosum*	Sri Lanka
PD 1418	M. Goto, Str 1-2	1	3	*Strelitzia reginae*	Japan
PD 1425	A.C. Hayward, UQM, 006 S	1	3	*Xanthium pungens*	Australia
PD 1682	V. Sardsud	1	3	*Zingiber officinale*	Thailand
PD 508	= NCPPB 1579	1	4	*Zingiber officinale*	Hawaii
PD 1426	A.C. Hayward, UQM, 009 S	1	4	*Zingiber officinale*	Australia
PD 1455	A. Kelman UW 197, ex Zehr IA 1	1	4	*Zingiber officinale*	Philippines
PD 1459	A. Kelman UW 359, ex He	1	4	*Zingiber officinale*	China

T = Type strain; NT = Neotype strain; PVRS = pathotype strain; NP = neopathotype strain

a) received as race 2, biovar 1; b) received as race 3, biovar 2

1) PD, culture collection, Plant Protection Service, Wageningen, the Netherlands

2) NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.

3) LMG, culture collection, Laboratorium Microbiologie, Gent, Belgium

4) UQM, culture collection, University of Queensland, Dept. of Microbiology, Queensland, Australia

5) IBSBF, culture collection, Phytobacteriology Section, Instituto Biologico, Sao Paulo, Brazil

6, 7) NHI, NZRM, National Health Institute and N. Zealand reference culture collection, medical section, Porirua, N. Zealand

8) MAFF, National Institute of Agrobiological Resources, Dept. Genetic Resources II, Yatabe, Japan

9) UW, culture collection, University of Wisconsin, Madison , U.S.A.

10) DAR, Australian National Collection of Plant Pathogenic Bacteria, Rydalmere, Australia

Results

Confirmation of identity of P. solanacearum strains by conventional tests

Biovardetermination. Classification into biovars, based on acid production from six carbohydrates is mentioned in Table 9b. Where the biovar of strains already had been determined the present classification matched completely, except for PD 1654 (see below). Biovars were not related to a particular race, except biovar 2, which was confined to race 3.

Race determination. Results of pathogenicity tests and race determination are presented in Table 9b and 10. Where the race of strains already had been determined the present classification matched completely, except for PD 1654 and 1453. The former strain was received as race 3, biovar 2, but proved to be race 1, biovar 3. The latter was received as race 2, biovar 1, but proved to be race 1, biovar 1. This strain was weakly pathogenic on tobacco and *Zingiber* and non-pathogenic on *Musa acuminata.*

Using tobacco inoculation and HR on tobacco, virulent strains of race 1 and 3 were easily separated (Table 10). An exception was strain PD 1434, being highly pathogenic on tobacco, but causing a typical HR (rapid collaps of infiltrated intercostal leaf tissue, within 15h after inoculation).

TABLE 10. Results of pathogenicity tests and hypersensitivity test on tobacco

Host	Reaction pattern of Race 1	Race 2	Race 3	*Zingiber* strains
tobacco 'White Burley' (stem inoculation)	+[1]	-	-	±/-
tobacco 'White Burley' (hypersensitivity test)	N	HR	HR[7]/Y	N
eggplant 'Black Beauty'	+[2]	+	+[3]	+
tomato 'Moneymaker'	+[2]	-/±	+[4]	-/±
Zingiber officinale	-[5]	-	-	+
Musa acuminata	-	+[6]	-	-

+ = clear wilting symptoms; ± = doubtful reaction; - = negative reaction;
N = necrosis after 48 h plus wilting of infiltrated leaf or complete plant;
HR = typical hypersensitivity reaction after 24 h; Y = yellowing of infiltrated area
1) Weakly virulent race 1 strains weak symptoms only.
2) Strains tested: PD 1449, 1457, 1645-47, 1650-52, 1654-55
3) Strains tested: PD 427, 1100, 1649
4) Strains tested: PD 427, 1100, 1645, 1649, 1651, 1657
5) Strain PD 1453 weakly positive
6) Strain PD 1446 from *Heliconia* only slight distortion symptoms
7) Majority of race 3 strains HR positive

Avirulent race 1 strains (PD 511, 1410, 1454, 1458) could not be separated from race 3 strains which caused a typical HR. These avirulent strains produced typical HR and no symptoms after inoculation in tobacco. Virulent race 3 strains could be discriminated from avirulent race 1 strains by inoculation in tomato and/or eggplant. Unfortunately avirulent strains of race 1 and 3 (and of any other race) cannot be separated using the host-inoculation system. Race 2 strains and *Zingiber* strains

(described as race 4) were discriminated from race 1 and 3 by symptom formation on *Musa acuminata* and *Zingiber officinale*. The only race 2 strain from *Heliconia* (PD 1446) which was tested caused only some distortion symptoms on *Musa acuminata*. One race 1 strain (PD 1453) from *Heliconia* was found to be weakly pathogenic on *Zingiber*, producing mild symptoms after prolonged incubation only. This strain was not pathogenic on *M. acuminata*.

Infraspecific classification of P. solanacearum strains, using fatty acid analysis

When performing principal component analysis (PCA) with fatty acid patterns obtained from the 69 *P. solanacearum* strains four clusters could be determined (Fig.4). The mean percentages of fatty acids of the four clusters are given in Table 11. Three of these clusters correspond to the known races 1, 2 and 3. The fourth cluster consisted of five deviating race 1, biovar 1 strains from the U.S.A.: PD 1447, 1449 and 1650, *L. esculentum*; PD 1456, *Nicotiana* spec. and PD 1655, *S. melongena*. They differed from other strains in lower percentages of 16:1 cis9 and 18:1 fatty acids and higher percentages of 16:0 and cyclic 17:0. These strains behaved similar in repeated experiments. There were no apparent differences in growth rate on agar media, colony morphology or pigment production with normal race 1 strains. From PD 1650 the avirulent form (non-slimy colony type) was obtained. In fatty acid analysis this avirulent form had the normal race 1 fatty acid pattern. *Zingiber* strains (described as race 4) did not form a separate cluster. Three biovar 4 strains were found in the race 1 cluster, a biovar 3 strain (PD 1682) from Thailand behaved differently from all other race 1 strains. The 16:0 2OH and 16:1 2OH acids could not be detected in this strain during repeated testing at different times. Race 2 strains showed a less discrete cluster than race 1 and 3, which is clear from the 2-dimensional plot in Fig. 4. *Musa* strains clustered well, but the *Heliconia* strain was more apart.

Using cluster analysis essentially the same subdivision as with PCA was found. In total six clusters and four separate strains were detected (Fig. 5). Cluster I (deviating race 1 strains), III (race 2 *Musa* strains), VI (race 3) and strains 2 and 4 were also discriminated in PCA. Cluster IV consisted of race 1 strains from *Zingiber* (biovar 4) and one of pepper, tomato and *Xanthium*. Cluster II and V contained all the other race 1 strains. Strain 1 of Fig. 5 had a higher percentage of 18:1 and a lower percentage of 16:0 acids.

When comparing the profiles of the three races of *P. solanacearum* it appeared that race 2 and 3 are more closely related to each other than to race 1 (Fig. 6). When comparing them with the closely related *P. pickettii*, race 1 was most closely related and the deviating race 1, biovar 1 strains most distantly related to *P. pickettii* (Fig. 6).

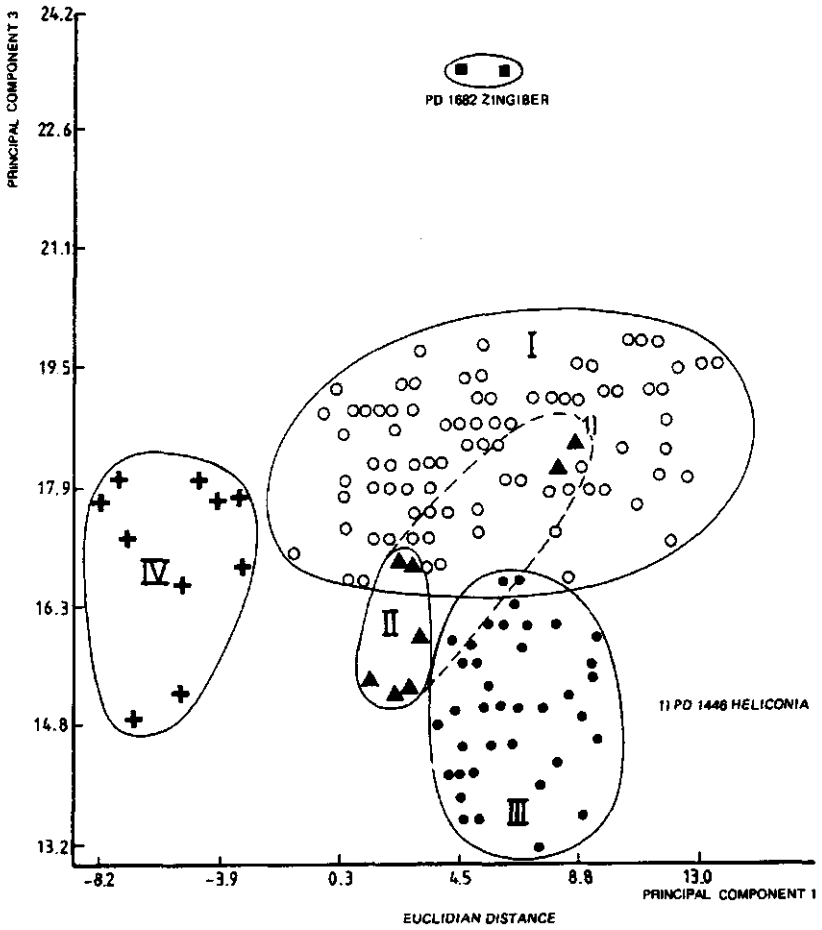

Fig. 4. Two-dimensional plot of principal component analysis of 69 strains of *P. solanacearum* (tested in duplo) showing subdivision into four clusters. Cluster I corresponds to race 1 strains, cluster II to race 2 strains, cluster III to race 3 strains and cluster IV to five deviating race 1, biovar 1 strains from the U.S.A.
N.B. this plot is a two-dimensional picture of a multivariate analysis, the clusters being more discrete than they seem to be in the figure.

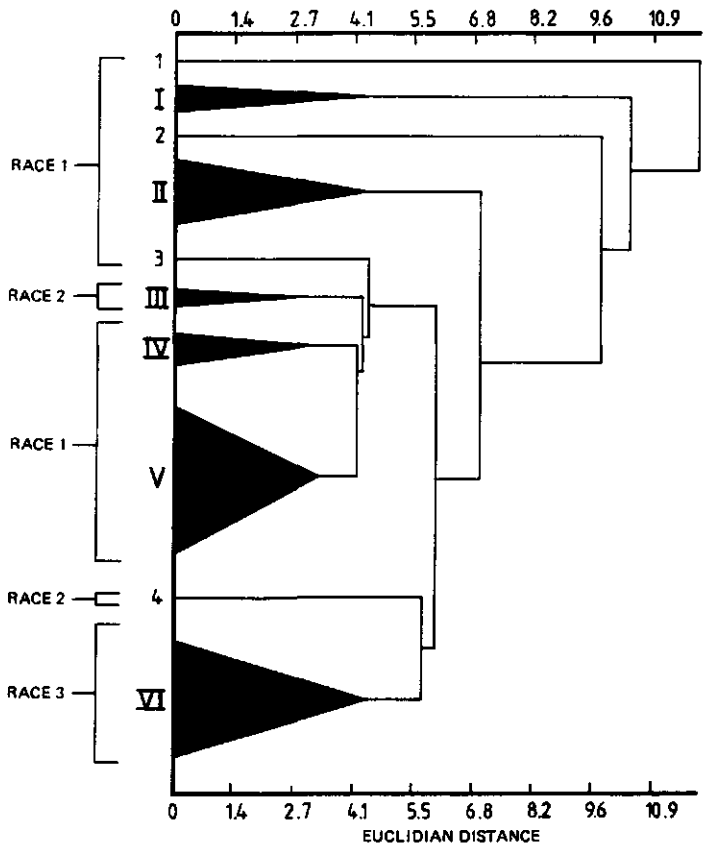

Fig. 5. Dendrogram showing relationships of 69 *P. solanacearum* strains, obtained in cluster analysis. Cluster I, five deviating race 1, biovar 1 strains from the U.S.A.; cluster II, 9 race 1 strains from miscellaneous hosts: PD 278, 1257, 1414, 1419, 1432, 1433, 1454, 1573, 1646; cluster III, race 2 *Musa* strains; cluster IV, 3 *Zingiber* race 1 strains and PD 1417, 1425, 1438 from other hosts; cluster V remaining race 1 strains; cluster VI, race 3 strains. Strain 1- PD 1652, race 1; 2 - PD 1682 race 1; 3 - PD 1436, race 1; 4- PD 1446, race 2, *Heliconia*.

A reference library of fatty acid patterns of the three races was constructed using PCA, enabling race identification of unknown strains.

TABLE 11. Whole cell fatty acids of race 1, 2 and 3 and deviating race 1, biovar 1 strains[1] of *Pseudomonas solanacearum*

Fatty acid	race 1 (42)[2]	race 2 (4)	race 3 (19)	deviating race 1, biovar 1 (5)
Saturated				
14:0	4.2[3](0.2)[4]	4.5(0.5)	4.9(0.3)	4.4(0.2)
15:0*	0.1(0.2)	-	0.2(0.3)	0.5(0.6)
16:0	26.1(2.2)	26.3(0.5)	23.4(1.4)	30.7(1.4)
17:0*	0.4(0.5)	0.1(0.2)	0.2(0.3)	0.5(0.5)
18:0*	0.5(0.3)	0.4(0.4)	0.1(0.2)	0.3(0.3)
Unsaturated				
15:1 iso*	-	-	-	0.2(0.2)
15:1 B*	0.1(0.3)	-	0.1(0.2)	0.2(0.4)
16:1 cis 9	25.5(2.1)	27.4(2.7)	27.7(1.9)	22.7(0.5)
18:1	20.7(2.4)	18.0(1.8)	19.2(1.1)	13.6(1.9)
Hydroxy				
14:0 3OH	7.2(0.5)	7.4(1.2)	7.9(0.4)	7.2(0.4)
16:0 2OH	0.9(0.3)	0.8(0.4)	0.7(0.2)	1.3(0.2)
Hydroxy unsaturated				
16:1 2OH	4.1(1.3)	6.3(0.8)	5.7(0.7)	3.9(1.0)
18:1 2OH	5.4(0.8)	5.1(0.3)	4.5(0.3)	4.5(0.4)
Cyclo				
17:0 cyclo	4.5(1.8)	3.7(1.3)	5.4(1.2)	9.4(0.7)
19:0 cyclo C11-12*	0.2(0.3)	-	0.1(0.2)	0.5(0.4)

* 50% or less of the strains positive; - = not detected

1) all strains tested in duplicate; 2) number of strains tested; 3) mean percentage; 4) standard deviation

TABLE 12. Results of biochemical tests with different *Pseudomonas* spp. used in this study

Test	*P. caryophylli*	*P. cepacia*	*P. gladioli*	*P. pickettii*	*P. solanacearum*
Arginine dihydrolase	-[1]	-	-	-	-
Denitrification	+	-/w[2]	-	+	+/-[7]
Growth at 40°C	+	+	+	+	-[8]
Gelatin hydrolysis	-	+[3]	+	-[6]	-
Starch hydrolysis	-	-	-	-	-
Acid from:					
D-xylose	+	d	+	+	+
meso-tartrate	+	+	+	+	-[9]
trehalose	+	+	+	-	+[10]
sucrose	+	+	+	-	+
Growth on:					
D-(tartrate)	-	-/+[4]	+	n.d.	n.d.
Oxidase reaction	+	+	+/-[5]	+	+
Catalase	+	+	+	+	+
O&F	+/-	+/-	+/-	+/-	+/-

+ = positive; - = negative; d = doubtful; n.d. = not done

1) I failed to obtain a positive reaction with the only strain (NCPPB 2151; type strain) used

2) PD 1500,1501 weakly positive

3) PD 1499 negative

4) PD 959, 1496, 1497, 1499, 1500 positive; PD 961, 1520-21 weakly positive

5) PD 981, 1502, 1504, 1507-08, 1517 negative

6) PD 1514, 1518 delayed positive

7) Only a few strains tested: PD 275, 1261 positive; PD 1256, 1100 negative

8) PD 279, 1429 positive

9) PD 1419, 1421 positive

10) PD 1260-63, 1452 negative

Fatty acids of P. solanacearum in relation to those of other Pseudomonas spp. of ribosomal RNA group II

The representation of total fatty acids found in 69 strains of *P. solanacearum* (Table 13) shows that ca.76% consisted of the straight chain saturated and mono-unsaturated fatty acids. About 17% were hydroxy acids, 14:0 3OH being the only quantitatively important saturated hydroxy acid. Cyclic acids were present in ca.6%. Fatty acids mentioned in Table 5 were present in more than 90% of the *P. solanacearum* strains, except 15:1 B (present in 16 strains), 15 :0 (22), 17:0 (30), 19:0 cyclo c 11-12 (25) and 18:0 (44). These minor acids were not used in statistical analysis.

To evaluate the "trueness" of fatty acid profiling in infraspecific classification it was compared to the relationships found within ribosomal RNA group II by DNA-DNA hybridization studies of Palleroni et al. (1973). Five out of the seven well described species of rRNA group II were compared, strains used are mentioned in Table 9a. Table 12 shows the results of verifying identity of strains by conventional tests.

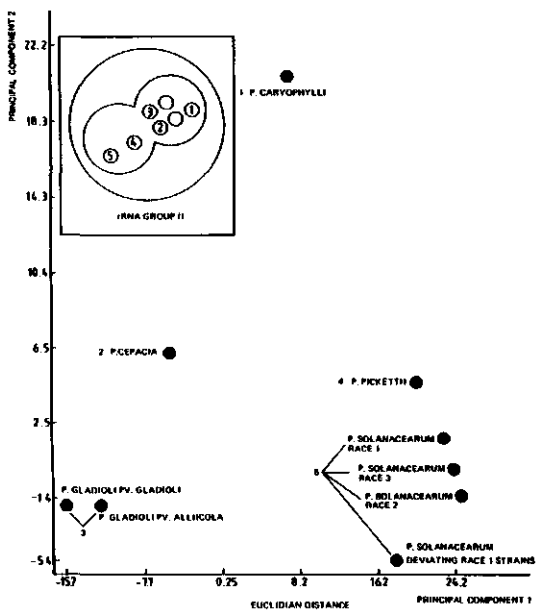

Fig. 6. Two-dimensional plot of principal component analysis of fatty acid patterns of five species of ribosomal RNA group II, showing intraspecific relationships. These relationships are well correlated to those established in DNA-DNA homology studies of Palleroni et al. (1973) (insert).

In Table 13 mean percentages of fatty acids found in the five species are presented. When these profiles were studied in PCA (Fig. 6) they yielded a taxonomic pattern very similar to the one obtained by DNA-DNA hybridizations (shown as an insert of Fig. 6). Most discriminative fatty acids between species were 16:1 cis 9, 18:1 and 19:0 cyclo C11-12.

P. pickettii and *P. solanacearum* were very similar in fatty acid pattern, but could be easily discriminated from the other species by the differences in percentages of the fatty acids 14:0 3OH, 16:1 cis 9, 16:0 2OH, 16:1 2OH, 18:1 2OH, 17:0 cyclo and 19:0 cyclo C11-12. Pathovars of *P. gladioli* could be separated by 10:0 3OH and 18:1 acids.

TABLE 13. Whole cell fatty acids of *Pseudomonas* spp[1] used in this study

Fatty acid	*P. caryophylli* (1)[2]	*P. cepacia* (18)	*P. gladioli* pv. *gladioli* (5)	*P. gladioli* pv. *alliicola* (5)	*P. pickettii* (10)	*P. solanacearum* (69)
Saturated						
10:0	-	-	-	0.5(0.6)	-	-
14:0	3.5(0.1)	4.1[3](0.1)[4]	4.3(0.2)	4.3(0.3)	4.5(0.3)	4.4(0.4)
15:0	0.3(0.04)	t	-	t	t	0.2(0.3)
16:0	20.6(0.03)	23.0(4.2)	23.4(1.6)	24.3(4.2)	24.1(1.0)	25.6(2.6)
17:0	0.35(0.02)	t	t	0.8	0.3(0.2)	0.3(0.5)
18:0	1.0(0.01)	1.3(0.4)	1.7(0.1)	1.3(0.6)	0.6(0.4)	0.4(0.3)
Unsaturated						
15:1B	-	-	-	-	-	0.1(0.3)
16:1 cis 9*	7.8(0.03)	6.2(2.3)	2.1(0.8)	2.9(3.6)	23.0(2.2)	26.0(2.4)
16:1C	-	t	-	-	t	-
18:1[5]*	35.2(0.2)	19.2(9.3)	8.3(2.7)	4.9(0.4)	22.9(1.4)	19.7(2.7)
Hydroxy						
10:0 3OH	-	-	t	2.8(2.4)	-	-
14:0 2OH	-	t	-	-	0.4(0.3) -	-
14:0 3OH*	4.3(0.1)	5.1(0.7)	4.5(0.3)	4.9(0.4)	7.6(0.7)	7.3(0.6)
16:0 2OH	2.0(0.2)	1.9(0.5)	2.6(0.4)	2.3(0.8)	0.5(0.7)	0.9(0.4)
16:0 3OH*	4.4(0.1)	5.2(0.4)	5.6(0.4)	5.9(0.4)	-	-
Unsaturated hydroxy						
16:1 2OH*	2.0(0.2)	1.2(0.5)	1.3(0.4)	1.1(0.6)	4.0(1.0)	4.6(1.4)
18:1 2OH*	2.6(0.04)	2.8(0.7)	3.2(0.5)	2.8(0.9)	5.0(0.6)	5.1(0.8)
Cyclo						
17:0 cyclo*	7.3(0.03)	15.6(4.8)	18.9(1.2)	18.3(3.3)	5.9(1.4)	5.0(2.0)
19:0 cyclo						
C11-12*	8.0(0.7)	14.4(4.4)	23.5(2.8)	18.6(6.3)	1.1(0.9)	0.2(0.3)

1) all strains tested in duplicate; 2) in brackets number of strains tested; 3) mean % of fatty acid; 4) standard deviation. 5) This acid may be 18:1 cis 9, trans 6 or trans 11, due to closely eluting methylesters of these forms.

* = discriminative fatty acid; t = trace, present in only a few strains.

Discussion

This study demonstrates that fatty acid analysis can be used to distinguish amongst races 1, 2 and 3 of *P. solanacearum* when applying principal component analysis. Using cluster analysis discrimination was more difficult. Fatty acid analysis is a powerful addition to conventional pathogenicity tests, because avirulent race 1 strains and those of race 3 can be distinguished. Race determination by conventional tests (pathogenicity, HR, colony morphology) is more laborious and liable to variability and error. This is not only due to the problem with avirulent strains, but also to frequent changes in colony morphology (French & Sequira, 1970) and to changes such as when pathogenic race 1 strains give a typical HR (Granada & Sequira, 1975; He et al, 1983). One such a pathogenic, HR-positive strain (PD 1434 from *L. esculentum*) was found in this study. Moreover we found other race 1, biotype 3 and 4 strains giving a typical HR, but they also showed the normal yellowing and wilting of the infiltrated leaf. Another indication of the power of fatty acid analysis was the identification of strains PD 1453 and 1654 as race 1, while they were received as race 2 and 3, respectively. This identification was confirmed by pathogenicity tests.

Recently RFLP analysis has also found to be useful for race determination (Cook et al., 1989). However, when strains are found with an unknown hybridization pattern, the race has to be determined in the conventional way. In comparison with RFLP analysis, fatty acid analysis is able to determine the race of unknown strains.
In PCA of fatty acid patterns race 2 and 3 were found to be slightly more related to each other than either is to race 1. Indications that this is true were also found in serology (Morton et al., 1966) and in RFLP analysis (Cook, et al., 1989). Cook et al. (1989) even went so far to lump race 2 and 3 and race 1, biovar 1 strains into one group and to claim a possible South American origin for this group. There are indications that this is feasible, especially for race 2. It has been found on triploid banana and on *Heliconia* spp. in South America only, except for the Philippines, where it most likely has been imported with planting material (Buddenhagen, 1986). The low temperature race 3, occurring naturally solely on potato and tomato, is thought to have been spread exclusively by potato trade from South America. The homogeneity in fatty acid and RFLP analyses of race 3 speak for the hypothesis of a South American origin, the findings of this race in virgin soils of Sri Lanka (Seneviratne, 1969) and perhaps its finding in Japan (Katayama & Kimura, 1984) against it.

In fatty acid analysis a group of five race 1, biovar 1 strains showed a distinct pattern from other race 1 strains. If there is any correlation between this deviating pattern and a pathogenic specialization, such as described for southern U.S.A. strains

by Kelman & Person (1961), is not known. Remarkably the avirulent form of one of the deviating strains showed a normal race 1 fatty acid pattern. From this fact a relation between pathogenicity and fatty acid composition can be suspected. More strains of race 2 should be studied to see if they form a homogeneous cluster or that different (pathogenic) types exist as described e.g. by French & Sequira (1970). *Zingiber* strains have been described as race 4 on the basis of a more limited host range than race 1 strains. Fatty acid patterns did not indicate the biovar 4 strains of this host to be different from race 1 strains, only PD 1682, a biovar 3 strain from Thailand was separate. In RFLP analysis biotype 4 *Zingiber* strains were also placed in race 1 (Cook et al., 1989). I did not study *Morus* strains, described as race 4, too (He et al., 1983), they were also placed in race 1 with RFLP analysis.

Fatty acid patterns of *P. solanacearum* did not correlate with the biotypes, described by Hayward (1964), except for biovar 2 which was confined entirely to race 3. Some exceptions to this rule have been described (Buddenhagen, 1986; Prior & Steva, 1990). The possibility of discriminating between so called pathotypes of *P. solanacearum* using fatty acid analysis is not very likely. RFLP analysis is more powerful in this matter (Cook et al., 1989). Perhaps polyacrylamide-gelelectrophoresis using whole cell proteins is a useful tool here, though biovar 1, race 1 strains from different hosts showed a very similar pattern (Janse, unpublished). Pathotypes seem not to be so much geographically determined as they may already occur in one infected field (McLaughlin & Sequira, 1989; Okabe & Goto, 1961).
In conclusion it can be stated that races of *P. solanacearum* are not 'mainly subjective groupings of pathotypes' (Buddenhagen & Kelman, 1964). There is now evidence from work on DNA fingerprinting (Cook et al, 1989), fatty acid composition (this article) and many studies on pathogenic behaviour that these races are more natural taxonomic entities than expected. On the other hand the nomenclatorial term 'race' unfortunately is vague and obsolete in the field of bacteriology. He (1986) suggested that strains could be divided into the pathovars *batatae*, *zingiberi*, *urtici*, *mori* and (race 3 strains) pv. *potatus*. For race 1 strains, infecting Solanaceae pv. *solanacearum* was suggested.

Indeed it would be useful to replace 'race' by the term 'pathovar'. Pathovar is used here in the meaning of 'pathogenic to (specialized on) one or more hosts under natural conditions', where fatty acid and RFLP characters should have equal and biochemical and morphological characters less taxonomic weight - see Dye et al., 1980. By closely following He(1986) pv. *solanacearum* for race 1 strains, pv. *musae* for race 2 and pv. *potatus* for race 3 could be suggested. Whether the other pathovars mentioned by He deserve this rank needs further elaboration. Formal pathovar establishment should include more strains than used in this study and include the indication of pathovar type

strains (Dye et al., 1980). The use of the term 'pathotype' for strains which differ in virulence to one or more hosts (so called epidemic or ecological strains) should be discouraged. Pathotype as a nomenclatorial term is in bacteriology equal to pathovar and has been abandoned. These specialized strains could be labelled by a number or a name, p.e. *P. solanacearum* pv. *solanacearum* (RFLP group 5 strain).

The overall fatty acid pattern of *P. solanacearum*, *P. cepacia*, *P. gladioli* pv. *gladioli* and *P. caryophylli* was very much the same as determined by Oyaizu & Komagata (1983), using another method and other growth and chromatographic conditions. I did not detect 19:0, but found 16:1 2OH and 18:1 2OH to be present between 1 and 5%.

When fatty acid patterns of *P. solanacearum* were compared with four other species of rRNA homology group II a taxonomic relationship was found similar to the one established in DNA-DNA homology studies (Ballard & al., 1970; Palleroni et al., 1973). *P. pickettii*, a bacterium found in human clinical bacteriology, was most closely related to *P. solanacearum* and both were more distantly related to *P. cepacia*. This confirms earlier work by Palleroni et al. (1973) and Ralston et al. (1973). The two pathovars of *P. gladioli* could also be separated by fatty acid analysis. This shows once more that fatty acid analysis can be a supportive tool in distinguishing and classifying pathogenic types within plant pathogenic bacterial species.

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II.4 CLASSIFICATION OF FLUORESCENT SOFT ROT *PSEUDOMONAS* BACTERIA, INCLUDING *P.MARGINALIS* STRAINS, USING WHOLE CELL FATTY ACID ANALYSIS

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Summary

In this study the taxonomy of the opportunistic plant pathogenic soft rot *Pseudomonas* bacteria has been investigated. This also in order to clarify the status of the species name *Pseudomonas marginalis* which is used either for soft rot strains resembling *P. fluorescens* biovar 2 (= *P. marginalis* sensu stricto) or for any fluorescent, oxidase positive soft rot pseudomonad (= *P. marginalis* sensu lato). It was also tried to verify the possible soft rot activity of *P. aeruginosa*, isolated from plant, animal and man.

In total 164 saprophytic and pathogenic strains, including 15 reference strains were studied. Conventional physiological and biochemical tests, pathogenicity tests on potato disks and chicory heads and whole cell fatty acid analysis (FAA) were applied. Of the non-reference strains 24 were identified phenotypically as *P. aeruginosa*, 81 with some difficulty, due to many variations, as biovars, intermediates of these biovars or new forms of *P. fluorescens*, two as *P. aureofaciens*, two as *P. cichorii*, 11 as *P. putida*, 21 as unknown fluorescent pseudomonads, two as *P. stutzeri* and seven as *P. viridiflava*. Oxidase positive soft rot strains could not be distinguished by biochemical tests from *P. fluorescens*, *P. putida* or *P. aureofaciens*. There was only a low correlation between pectolytic activity and ability to cause soft rot. In multivariate analysis of fatty acid patterns four clusters could be distinguished, viz.: one heterogeneous (super) cluster containing strains of *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. putida* and *P. tolaasii* and strains received as *P. marginalis;* one cluster comprising the oxidase negative *P. syringae* and *P. viridiflava* and the related oxidase positive *P. cichorii;* one containing *P. stutzeri* and *P mendocina;* one tight cluster with all but one *P. aeruginosa* strains.

All oxidase positive soft rot strains were found in the *P. fluorescens* supercluster and all the oxidase negative ones (phenotypically *P. viridiflava)* in the cluster containing *P. syringae*, *P. viridiflava* and *P. cichorii*. Within the *P. fluorescens*

supercluster there were no clusters separating soft rot strains *(P. marginalis* sensu stricto or sensu lato), they were found scattered among saprophytic strains. The absence of clustering of soft rot strains as a separate taxon in phenotypic or fatty acid analysis renders the species name *P. marginalis* artificial and its use should be abandoned. The taxonomic implications of finding a continuum of strains within the P. fluorescens complex (including *P. aureofaciens, P. chlororaphis, P. putida* and *P. tolaasii)* using FAA or phenotypic tests is discussed, also in relation to future naming of soft rot pseudomonads.

The possible soft rot activity of *P. aeruginosa* could not be confirmed, only a few strains occasionally caused some necrotizing action on plant tissue. Finally pathogenic, soft rot-negative strains of *P. fluorescens,* causing bacterial leaf stripe of *Iris* spec. are described.

Introduction

Fluorescent *Pseudomonas* bacteria causing soft rot in a large number of plants are an important factor in pre- and post harvest crop loss (Lund 1983; Wells & Liao 1987). One of the earliest descriptions of the relation between soft rot (of potato) and a fluorescent *Pseudomonas* bacterium was by Schuster in 1912. He named the organism *Bacillus xanthochlorum,* which was renamed *Pseudomonas xanthochlora* by Stapp in 1928. Schuster compared it with the saprophytic *Bacillus fluorescens liquefaciens (= Pseudomonas fluorescens)* which he found to be very similar. Due to description according to standards of that time and lack of authentic strains, we now ignore its real identity.

In 1918 Brown described a bacterial disease of lettuce in Kansas, USA, showing blight of leaf margins and black veins. She named the causal organism *Bacterium marginale,* renamed *Pseudomonas marginalis* by Stevens in 1925. *B. marginale* was fluorescent, aerobic, denitrifying and had a relatively low optimum growth temperature (25-26°C). In later years this organism has been reported on lettuce in various countries and also on *Cichorium endivia* and *C. intybus* (Stapp, 1956). In Germany it was described as *C. intybi,* causing soft rot (Stapp, 1934).

Lelliott et al. (1966) were the first to compare in greater detail a number of fluorescent soft rot bacteria, including six strains named *P. marginalis,* with pathogenic and saprophytic pseudomonads associated with plants. They found two groups of oxidase positive soft rot organisms, based on 12 tests including soft rot of potato disks. One group (IVa) included *P. marginalis* strains, *P. pastinacae* (a pathogen

related to *P. marginalis*, described by Burkholder in 1960 and renamed *P. marginalis* pv. *pastinacae* by Young et al., 1978), and some nitrate reduction negative strains. The other group (IVb) differed essentially in negative reactions for levan formation from sucrose and sucrose fermentation. Lelliott et al. (1966) also described a group of oxidase negative soft rot organisms (group II) which included *P. viridiflava*, a pathogen described from bean by Burkholder in 1930 and some related strains.

In 1930 Clara described a wet rot and leaf spot of tobacco in the Philippines, caused by a denitrifying fluorescent bacterium with a high temperature optimum (37°C) which he named *Phytomonas polycolor*. This oxidase positive organism was later shown to be identical to *Pseudomonas aeruginosa* (Elrod & Braun, 1942). *Pseudomonas aeruginosa* had already been known as a secondary pathogen for man and animals widely distributed in nature and distinguished by the presence in most strains of a blue diffusible pigment, pyocyanin. Many years later *P. aeruginosa* was reported as the cause of an internal brown rot of onion in Australia (Cother et al., 1976), but its plant pathogenic properties are not well established.

In 1966 Stanier et al. published their extensive phenotypic study of the genus *Pseudomonas*. They also included two strains of *P. marginalis* from lettuce and *Dahlia*. In the fluorescent pseudomonads they recognized the species *P. aeruginosa* (very homogeneous), *P. fluorescens* (very heterogeneous) and *P. putida* (heterogeneous). The 94 strains of *P. fluorescens* were further subdivided in the biotypes A-G. The *P. marginalis* strains appeared to be phenotypically indistinguishable from saprophytic strains of biotype B. This intimate relationship was confirmed by Misaghi & Grogan (1969) and Sands et al. (1970) in nutritional studies. It was also confirmed by Pecknold & Grogan (1973) in DNA-homology studies, though the four *P. marginalis* strains used showed a variation of 17-59% homology with the biotype B reference strain. Biotypes A-C, F and G of *P. fluorescens* were later named biovar 1-5 and biovars D and E, *P. chlororaphis* (Johnson & Palleroni, 1989). Biotype G (= biovar 5) was used as a reservoir for non-biotypable strains, 18% of the strains was assigned to this biovar.

After the study of Stanier et al. (1966) soft rot pseudomonads have been described in many plants all over the world. Their classification and naming, however, has become very confusing for the following reasons: a) strains described as *P. marginalis* appeared to differ from *P. fluorescens* biovar 2 in their pectolytic activity and ability to cause soft rot only. These two factors were found to be present in many other oxidase positive fluorescent pseudomonads which could not be classified as biovar 2. These organisms were other biovars, intermediates or *P. putida* (Brocklehurst & Lund,

1981; Sands & Hankin, 1975; Shinde & Lukezic,1974; Vantomme et al., 1989; Wang & Kelman, 1987; Wells & Liao, 1987); b) The biovar concept of *P. fluorescens* has become less clear by the finding of many intermediates when more strains were studied (Molin & Ternstrom, 1986; Samson, 1982; Sands & Rovira, 1971); c) Due to a and b the position which was first elaborated by Cuppels & Kelman (1980) that *P. marginalis* should include any oxidase positive, fluorescent soft rot pseudomonad, became common practice; d) Due to c and other reasons many soft rot bacteria have been named *P. marginalis* based on marginal descriptions and without extensive testing.

The present status of *P. marginalis*, including *P. m.* pv. *pastinacae* and *P.m.* pv. *alfalfae*, isolated from discoloured alfalfa roots (Shinde & Lukezic, 1974) is thus far from clear. Whole cell fatty acid analysis (FAA) has been found to be a useful tool for discrimination between *Pseudomonas* bacteria at and below species level, including pathogenic varieties (Oyaizu & Komagata, 1983; Janse, 1991 a and b). Therefore we tried to clarify the status of *P. marginalis* and the taxonomic position of a larger collection of soft rot pseudomonads using this technique. FAA was used in combination with conventional biochemical tests and tests for pectolytic activity, potato soft rot and pathogenicity on chicory heads, also in order to study pathogenic properties of *P. aeruginosa*. In total 164 strains, including 15 reference strains, were studied.

In this article we report on 1) the confirmation of the heterogeneity of *P. fluorescens* and the homogeneity of *P. aeruginosa* in FAA and conventional tests; 2) the inclusion of all oxidase positive fluorescent soft rot pseudomonads in *P. fluorescens*, including *P. marginalis* strains; c) the clear distinction between *P. fluorescens* (incl. *P. putida, P. aureofaciens, P. chlororaphis* and *P. tolaasii)* and *P. aeruginosa* and other *Pseudomonas* spp. by FAA; d) the very limited plant pathogenic properties of *P. aeruginosa* and finally e) the description of soft rot negative *Pseudomonas fluorescens* strains pathogenic for *Iris*, causing bacterial leaf stripe.

Material and methods

Bacterialstrains. Table 14 lists the bacterial strains used, with their classification on the basis of phenotypic tests (see Table 15) performed by us. Most strains were isolated in our laboratory from plants showing symptoms of soft rot. Others were received from culture collections and colleagues from different parts of the world. From all species and pathogenic varieties tested, the type strain and pathotype strain

was used, except for *P. stutzeri*. All strains were lyophilized and routinely grown on nutrient agar (NA, Difco).

Physiological and biochemical tests. For identification of *Pseudomonas* bacteria at species and biovar level methods applied were according to Palleroni (1984) and tests (Table 15) were taken from his identification tables. Carbohydrates in minimal medium (Ayers et al., 1917) had a concentration of 0.1% w/v (except β-hydroxybutyrate: 0.5%) and they were filter sterilized. Geraniol (Sigma) was added as drops in the lid of a Petri dish and incubated in inverted position. Gelatin hydrolysis, arginine hydrolysis and potato soft rot were studied according to the methods of Lelliott et al. (1966). All carbon sources were tested in duplicate. Pectolytic activity was detected on the medium of Hildebrand (1971) at pH 5.5. Strains showing negative results on this medium were also tested at pH 8. Shallow pits in the pectin medium were recorded as weak, clear pits as positive. Ice nucleation activity (*Iris* strains) was tested according to Paulin & Luisetti (1978).

Pathogenicity test on chicory. Chicory heads *(Cichorium intybus* cv. Zoom) were used for testing pathogenicity, i.e. soft rot capacity. The chicory heads were placed in 1 m long plastic trays on a wet layer of perlite. Three to five plants were used per strain and 25 strains tested at a time. Inoculation was performed with a 23 G hypodermic needle, using a 10^7 cells.ml^{-1} suspension in sterile physiological buffered saline (PBS, pH 7.2) of a 24 h NA culture. Three to four inoculations were made in leaves of each plant and the suspension infiltrated to some extend in the parenchymatic tissue. Bacterial concentration was adjusted, using a BaSO4 standard (Kiraly, 1974). Control plants (4-8 per test series) were inoculated as described above, using sterile PBS. When in the first series strain PD 903 was found to cause severe soft rot, this strain served as a positive control in each subsequent series. After inoculation plants were wetted and trays covered with transparant polyethylene foil. Light from mercury lamps, c. 10.000 lux was given for 12 h a day. Observations were made up to 15 days after inoculation. Reisolations were performed at regular intervals from plants showing symptoms and identity confirmed by colony morphology on NA + 5% w/v sucrose and on King's medium B (King et al., 1954) and fatty acid analysis. *P. aeruginosa* strains showing symptoms on chicory or potato disks were retested.

One brown spot up to 2 cm in length on one chicory head was recorded as doubtful (d), several spots up to 2 cm on several heads as weak (w), when these spots were dry and necrotic as necrotic (N), several wet spots more than 2 cm on several heads as positive (+) and rapidly spreading slimy rot of head leaves as strong positive (++).

Pathogenicity test on Iris 'Apollo' with five P. fluorescens strains. Five *P. fluorescens* strains which were isolated from *Iris* spp. showing symptoms of

bacterial leaf stripe were inoculated into *Iris* 'Apollo' by the following procedure: 32 (2 x 16) plants per strain in wooden crates were scratched with a needle with some growth of a 48 h NA-culture. Inoculated plants were kept in a greenhouse at 18°C and after three weeks they received a cold treatment of four nights at - 2°C in a cold store. This cold treatment was applied because symptoms of bacterial leaf stripe were found to be prevalent in spring.

Fatty acid analysis. Bacteria were grown for 24 h at 28°C on Trypticase Soy Broth Agar, containing w/v 3% Trypticase Soy Broth (BBL) and 1.5% Bacto Agar (Difco). Harvesting, gas-chromatographic analysis using the Microbial Identification System (MIDI, Newark, DE, USA), reference library generation and principal component and cluster (UPGMA) analysis were performed as described by Janse (1991a). All strains were tested in duplicate.

Results

Identification of strains by conventional physiological and biochemical tests

Using discriminative tests from Palleroni (1984) which are mentioned in Table 15, the identification of 163 strains (PD 1549 not tested) is presented in Table 14. All strains were Gram-negative and showed only oxidative metabolism of glucose.

Twenty-five strains were identified as *P. aeruginosa* (PD 1549 was not tested, but was received as *P. aeruginosa* and it fell in the same fatty acid cluster as 23 other *P. aeruginosa* strains). These strains reacted very uniform and showed only one to two differences from the expected test reactions. We found strains usually geraniol-utilization negative and many strains lecithinase positive. Strains PD 277, 1351, 1584, 1601 en 1602 produced pyoverdin pigment only and no pyocyanin. Strains PD 1359, 1363, 1366, 1530 and 1550 produced a brown diffusible pigment. There were no significant differences between strains from man, animals or plants with the tests used. *P. aeruginosa* strains could be easily discriminated from all other strains on the basis of positive tests for pyocyanin, growth at 41°C and denitrification and negative tests for growth at 4°C and utilization of trehalose.

Contrary to the homogeneous *P. aeruginosa* strains, bacteria which were found to belong to the species *P. aureofaciens, P. fluorescens* and *P. putida* showed much more variation. Only with some difficulty 13 strains were placed in biovar 1 of *P. fluorescens,* 38 in biovar 2, 3 in biovar 3 and none in biovar 4. Seventeen strains could only be placed in the ill defined and variable biovar 5. Thirteen strains could not be assigned to any biovar, 11 were identified as *P. putida.* From the remaining strains

seven were similar to the oxidase-negative species *P. viridiflava* and 21 strains did not show similarity in test results to any known fluorescent *Pseudomonas* species. The type strain of *P. tolaasii* was identified as a *P. fluorescens* biovar 5 strain. The number of deviations from the species and biotype pattern *(P. aureofaciens, P. fluorescens, P. putida)* of Palleroni (1984) ranged from 0-6. These deviations related to almost any test, rendering identifications for this group in many cases difficult and arbitrary, if not doubtful. Discrimination of the *P. cichorii/ P. syringae/ P. viridiflava* group and *P. stutzeri* was easy.
Reactions of non-identified *Pseudomonas* strains are given in Table 2. Many strains (38) from plants with soft rot were identified as *P. fluorescens* biovar 2, including strains received as *P. marginalis*. Some of the latter, however, were identified as biovar 5 or *P. putida*. Twenty of the 38 biovar 2 strains showed a pink diffusible pigment, PD 571, 1242, 1393 and 1960 a brown diffusible pigment. Biovar 5 strains PD 1628 and 1630 showed a pink and brown pigment respectively. This pigment production was often but not always correlated with pectolytic and/or soft rot activity.

All reference type and pathotype strains could be positively identified, except *P. tolaasii (P. fluorescens* biovar 5) and the pathotype strain of *P. marginalis* pv. *pastinacae* (PD 1559) which differed substantially from the *P. fluorescens* biovar 2 pattern.

Strains from *Iris* showing symptoms of bacterial leaf stripe could be placed in *P. fluorescens*, but biotyping was not possible. Their reaction pattern is mentioned in Table 15. They were motile and showed 1-3 polar flagella, except PD 839 and 841, which were non-motile. *Iris* strains did not have ice nucleation activity.

TABLE 14. Strains used with their origin and pectolytic and pathogenic properties (classification according to this study).

Strain	Received from/ alternate numbers	Host/ Locality	Country	Deviations[a]	Pectolytic	Potato soft rot	Pathogenic chicory
Pseudomonas aeruginosa							
PD[1] 277	= ATCC[2] 15442[b]	animal room	?	0	-	-	-
PD 971	= NCPPB[3] 1965 (T)	?	?	1	-	-	-
PD 1279	A.H. Aziz	human blood	Malaysia	0	-	-	-
PD 1351	= RVCL[4] 32/1288	nasal swab, dog	UK	1	-	-	-
PD 1358	= RVCL 39/389	external ear, dog	UK	1	-	N	-
PD 1359	= RVCL mm/389	mastitis, cow	UK	1	-	-	N
PD 1360	= RVCL KD/389	mastitis, cow	UK	1	-	-	N
PD 1362	= RVCL 12/489	chestaspirate dog	UK	1	-	-	-
PD 1363	= RVCL 21/489	intestine, lizard	UK	1	-	-	-
PD 1366	= RVCL 13/489	?	UK	1	-	-	-
PD 1524	= PCM[14] 2062	?	Poland	1	-	-	-
PD 1525	= PCM 2058=ATCC27853	blood	USA	1	-	-	-
PD 1530	= WADA[5] 518	snake abcess	Australia	1	-	-	-
PD 1531	= WADA 515	sheep, green wool	Australia	1	-	-	-
PD 1532	= DAR[6] 35663	*Nicotiana rustica*	Australia	1	-	-	-
PD 1533	= DAR 41301	*Allium cepa var. cepa*	Australia	1	-	-	-
PD 1549	= IBSBF[7] 517	*Nicotiana tabacum*	?	tested only by FAA			
PD 1550	= IBSBF 612	*Elaeis guineensis*	Brazil	2	-	-	-
PD 1568	= CN[8] 6585	cystic fibrosis, human	UK	2	-	N	-
PD 1569	= LMG[9] 5032	*Chrysanthemum sp.*	USA	2	-	N	-
PD 1577	= CN 8751	*Gtomphadorhina sp.*	?	2	-	-	-
PD 1583	= LMG 6855	*Hordeum vulgare* roots	Belgium	2	-	-	-
PD 1584	= LMG 5032	*Zea mays*	USA	1	-	N	-
PD 1601	= CCM[15]) 5707	clinical material	Czechoslovakia	2	-	-	-
PD 1602	= CCM 5708	clinical material	Czechoslovakia	1	-	-	-
PD 1603	= CCM 5710	clinical material	Czechoslovakia	1	-	-	-
Pseudomonas aureofaciens							
PD 1554	= LMG 5832 ti	soil	USA	2	-	-	-
PD 1585	= LMG 1245 = ATCC 13985 (T)	clay	Netherlands	1	-	-	-
PD 1673		*Cyclamen persicum*	Netherlands	2	+	-	-
Pseudomonas chlororaphis							
PD 1587	= LMG 6220 (T)	plate contaminant	?	2	-	-	-

TABLE 14. (continued)

Strain	Received from/ alternate numbers	Host/ Locality	Country	Deviations[a]	Pectolytic	Potato soft rot	Pathogenic chicory
Pseudomonas cichorii							
PD 377		*Chrysanthemum morifolium*	Netherlands	4	-	N	d
PD 468		*Chrysanthemum morifolium*	Netherlands	3	-	N	+
PD 478	= ATCC 10857 (T)	*Cichorium endivia*	Germany	3	-	N	d
Pseudomonas fluorescens, biotyping not possible							
PD 835		*Iris* 'Apollo'	Netherlands		-	-	-[c]
PD 836		*Iris* 'Siberica'	Netherlands		-	-	-[c]
PD 837		*Iris* 'Blue Magic'	Netherlands		-	-	-[c]
PD 838		*Iris* sp.	Netherlands		-	-	-
PD 839		*Iris* sp.	Netherlands		-	-	-
PD 840		*Iris* 'Apollo'	Netherlands		-	-	-[c]
PD 841		*Iris* sp.	Netherlands		-	-	-[c]
PD 842		*Iris* sp.	Netherlands		-	-	-
PD 843		*Iris* sp.	Netherlands		-	-	-
PD 1125		*Tulipa* 'White Dream'	Netherlands		-	-	-
PD 1179		*Cichorium intybus*	Netherlands		+	N	d
PD 1239		*Spathiphyllum* 'Prelude'	Netherlands		-	-	-
PD 1326		*Gerbera* sp.	Netherlands		-	-	-
Pseudomonas fluorescens biotype 1							
PD 1175		*Cichorium intybus*	Netherlands	2	-	-	-
PD 1243		*Anemone* sp.	Netherlands	3	+	+	+
PD 1364	= RVCL 50/389	tracheal wash (ox)	UK	3	-	-	-
PD 1537[d]	= DAR 41334	*Brassica oleracea*	Australia	4	-	-	-
PD 1552[d]	= IBSBF 532	*Lactuca sativa*	New Zealand	4	-	-	-
PD 1553[d]	= LMG 533	*Lactuca sativa*	USA	4	+	+	w
PD 1589	= LMG 1794 (T)	pre-filter water work tanks	UK	3	-	-	-
PD 1611		*Brassica oleracea*	Netherlands	3	w	N	+
PD 1613		*Freesia* 'Thetis'	Netherlands	3	w	N	N
PD 1714		*Pisum sativum*	Netherlands	2	+	++	d
PD 1765		*Allium porrum*	Netherlands	3	+	-	-
PD 1767		*Delphinium* sp.	Netherlands	3	+	-	-
PD 1775		*Apium graveolens*	Netherlands	1	+	++	+
Pseudomonas fluorescens biotype 2							
PD 101		*Cichorium intybus*	Netherlands	3	+	-	-
PD 203		*Lycopersicum esculentum*	Netherlands	2	+	+	w
PD 326		*Cichorium endivia*	Netherlands	2	+	+	w

TABLE 14. (continued)

Strain	Received from/ alternate numbers	Host/ Locality	Country	Deviations[a]	Pectolytic	Potato soft rot	Pathogenic chicory
PD 348		*Brassica oleracea*	Netherlands	3	-	-	-
PD 513[d]	= NCPPB 667 (PVRS)	*Cichorium intybus*	USA	2	+[e]	-	-
PD 545		*Allium sativum*	Netherlands	1	+	++	+
PD 559		*Papaver somniferum*	Netherlands	2	+	+	++
PD 571		*Cichorium intybus*	Netherlands	2	w	-	+
PD 671		*Cichorium intybus*	Netherlands	2	w	+	+
PD 705		*Chrysanthemum* sp.	Netherlands	4	w	+	d
PD 895		Bambasa groundnut	Mali	0	+	+	+
PD 980[d]	= NCPPB 667 (PVRS)	*Cichorium intybus*	USA	2	w[e]	-	-
PD 1052		*Solanum tuberosum*	Netherlands	1	w	+	+
PD 1065		*Primula* sp.	Netherlands	2	+	-	-
PD 1082		*Hyacinthus orientalis*	Netherlands	2	+	-	-
PD 1083		*Apium graveolens* var. *rapaceum*	Netherlands	4	+	+	d
PD 1105		*Aconitum napellus*	Netherlands	3	+	+	d
PD 1152		*Apium graveolens*	Netherlands	2	+	-	-
PD 1156		*Cichorium intybus*	Netherlands	2	+	N	d
PD 1228		*Solanum tuberosum*	Netherlands	1	+	+	+
PD 1242		*Cichorium intybus*	Netherlands	3	w	N	d
PD 1248		*Lactuca sativa*	Netherlands	4	w	+	+
PD 1393		*Dianthus caryophyllus*	Netherlands	2	-	-	-
PD 1404		*Nigella damascena*	Netherlands	3	w	++	+
PD 1430		*Agapanthus umbellatus*	Netherlands	1	+	-	-
PD 1441		*Lycopersicon esculentum*	Turkey	4	+	N	w
PD 1556[f]	= LMG 5040	*Medicago sativa*	USA	4	-	-	-
PD 1557[d]	= LMG 2210 = NCPPB 667 (PVRS)	*Cichorium intybus*	USA	4	w[e]	-	-
PD 1559[g]	= LMG 2238 (PVRS))	*Pastinaca sativa*	USA	6	+	-	-
PD 1560[g]	= LMG 5044	*Pastinaca sativa*	USA	3	+	N	N
PD 1594	= LMG 5177	*Phaseolus vulgaris*	UK	1	+	-	-
PD 1595	= LMG 5180	*Hyacinthus orientalis*	UK	3	+	-	-
PD 1600[f]	= LMG 2214 (PVRS)	*Medicago sativa*	USA	3	+	-	-
PD 1678		*Cichorium intybus*	Netherlands	0	+	+	+
PD 1702	= CFBP[10] 1968	*Apium graveolens*	Italy	4	+	++	+
PD 1712		*Iris germanica*	Netherlands	3	+	++	d
PD 1761		*Beta vulgaris*	Netherlands	2	+	N	-
PD 1788		*Cichorium intybus*	Netherlands	4	+	+	d

TABLE 14. (continued)

Strain	Received from/ alternate numbers	Host/ Locality	Country	Deviations[a]	Pectolytic	Potato soft rot	Pathogenic chicory
Pseudomonas fluorescens biotype 3							
PD 506		*Cichorium endivia*	Netherlands	2	+	++	+
PD 1536	= DAR 33375	*Brassica oleracea*	Australia	4	+	-	-
PD 1588	= LMG 1244	polluted seawater	Denmark	0	-	-	-
Pseudomonas fluorescens biotype 5							
PD 1096		*Rheum rhabarbararum*	Netherlands		+	+	d
PD 1116		*Solanum tuberosum*	Netherlands		+	+	+
PD 1123		*Iris* sp.	Netherlands		-	-	-
PD 1174		*Crataegus* sp.	Netherlands		-	N	N
PD 1271		*Allium porrum*	Netherlands		-	-	-
PD 1272[d]	= Hayward 01060	*Brassica oleracea* var.*italica*	Australia		+	++	N
PD 1535	= DAR 26821	Rhizosphere of *Triticum*	?		-	-	-
PD 1570[d]	= MAFF[11] 03-06531	*Oryza sativa*	Japan		-	-	-
PD 1574[d]	= MAFF 03-01512	*Allium cepa*	Japan		+	-	-
PD 1590	= LMG 5167	water (tryptophan enrichm.)	USA		-	-	-
PD 1591	= LMG 5168	water (hydrocarbon enrichm.)	USA		-	-	-
PD 1598	= ICMP[12]2746	*Coriandrum sativum*	?		w	-	-
PD 1628	= J.W.Miller P89 4122-2	?	USA		-	-	-
PD 1630	= J.W.Miller P89 4121-2	*Dieffenbachia* sp.	USA		-	-	-
PD 1748		*Brassica oleracea*	Netherlands		+	N	+
PD 1749		*Coriandrum* sp.	Netherlands		+	+	+
PD 1757		*Ilex verticullata*	Netherlands		+	+	-
Pseudomonas gladioli pv. *gladioli*							
PD 981	= NCPPB 1891 (PVRS)	*Gladiolus* sp.	?		-	-	-
Pseudomonas mendocina							
PD 1822	= LMG 1223 = ATCC 25411(T)	soil	?		-	-	-
Pseudomonas putida							
PD 1193		*Cichorium intybus*	Netherlands	3	+	N	d
PD 1539	= DAR 26820	rhizosphere of *Triticum*	?	3	-	-	-
PD 1546[i]	= DAR 35663	*Nicotiana rustica*	Australia(?)	0	-	-	-
PD 1555[j]	= LMG 5850 = NCPPB 1876	*Cichorium endivia*	USA	3	-	-	-
PD 1561	= LMG 2257, biot. A (T)	soil	USA	3	-	-	-
PD 1571	= MAFF=03-01684	soil	Japan	5	-	-	-
PD 1596	= LMG 1246	?	?	4	-	-	-
PD 1597	= LMG 2259	*Hyphantria cunea*	Czechoslovakia	3	-	-	-

TABLE 14. (continued)

Strain	Received from/ alternate numbers	Host/ Locality	Country	Deviations[a]	Pectolytic	Potato soft rot	Pathogenic chicory
PD 1609	= NCPPB 1611[d]	*Rheum raponticum*	UK	3	+	-	-
PD 1785		*Lactuca sativa*	Netherlands	3	+	+	+
PD 1834	= WCS [13] 604	?	Netherlands	2	-	-	-
PD 1835[8]	= WCS 608	?	Netherlands	2	-	-	-
Pseudomonas species, fluorescent, not identifiable							
PD 123	= NCPPB 1465	*Fraxinus excelsior*	UK		-	-	-
PD 212	= NCPPB 2875	*Agaricus bisporus*	UK		-	-	-
PD 1054		*Cichorium endivia*	Netherlands		w	N	d
PD 1246		*Phalaenopsis sp.*	Netherlands		-	-	-
PD 1247		*Lactuca sativa*	Netherlands		-	-	-
PD 1270		*Cichorium intybus*	Netherlands		-	-	-
PD 1276		*Solanum tuberosum*	Belgium		+	-	N
PD 1534[k]	= DAR 54610	*Triticum durum*	USA		-	-	-
PD 1538[l]	= DAR 26771	*Agaricus bisporus*	Australia		-	-	-
PD 1566	= CN 6807	?	UK		-	-	-
PD 1567	= CN 6540	?	?		-	-	-
PD 1592[d]	= LMG 5175	*Musa* sp.	Uganda		+	+	w
PD 1599	= NCPPB 247	*Lactuca sativa*	USA		-	-	-
PD 1715		*Eremurus* sp.	Netherlands		+	N	w
PD 1752		*Pachipodium lameri*	Netherlands		+	N	+
PD 1760		*Aconitum* sp.	Netherlands		+	-	-
PD 1762		*Allium cepa*	Netherlands		+	++	w
PD 1763		*Hosta* sp.	Netherlands		+	++	+
PD 1771		*Saintpaulia*	Netherlands		w	-	-
PD 1836		*Samolus* sp.	Netherlands		-	-	-
Pseudomonas species, non-fluorescent, not identifiable							
PD 1586[n]	= LMG 5824	?	?		-	-	-
Pseudomonas stutzeri							
PD 1335	A.K. Mischra	oil exploration area	India	3	-	-	-
PD 1658		soil	Netherlands	5	-	-	-
Pseudomonas tolaasii							
PD 210[m]	= NCPPB 2192 (T)	*Agaricus bisporus*	UK		+	++	w
Pseudomonas syringae							
PD 184	= ATCC 19310 (T)	*Syringa vulgaris*	UK	0	-	N	d

TABLE 14. (continued)

Strain	Received from/ alternate numbers	Host/ Locality	Country	Deviations[a]	Pectolytic	Potato soft rot	Pathogenic chicory
Pseudomonas viridiflava							
PD 798		*Cichorium intybus*	Netherlands	1	w	w	++
PD 903		*Cichorium intybus*	Netherlands	o	w	+	++
PD 988	= NCPPB 635 (T)	*Phaseolus* sp.	Switzerland	4	+[e]	+	-
PD 1035		*Brassica oleracea*	Netherlands	3	+	N	d
PD 1151		*Brassica oleracea*	Netherlands	3	w	+	+
PD 1528	M. Goto Pv 21 12	?	Japan	5	-	-	-
PD 1529	M. Goto Pm 1973	?	Japan	4	+	N	-
PD 1551	= IBSBF 615	*Nicotiana tabacum*	?	5	-	-	-

+ = positive; ++ = strong positive; w = weak; d = doubtful; - = negative; N = only black (potato) or red (chicory) necrotic tissue degeneration without maceration; T = type strain; PVRS = pathotype strain.

a = number of differences with identification tables in Bergeys's Manual of Systematic Bacteriology, Vol. 1, pp. 141-199 (eds. N.R. Krieg & J.G. Holt). N.J. Palleroni Genus 1. *Pseudomonas* Migala 1894.

b = small colony type

c = pathogenic for *Iris* 'Apollo', causing symptoms of bacterial leaf stripe

d = received as *P. marginalis* pv. *marginalis*

e = positive at pH 8

f = received as *P. marginalis* pv. *alfalfae*

g = received as *P. marginalis* pv. *pastinacae*

h = received as *P. fluorescens* biotype F

i = received as *P. aeruginosa*

j = received as *P. fluorescens*

k = received as *P. aureofaciens*

l = received as *P. putida*

m = identified as *P. fluorescens*, biotype 5

n = received as *P. chlororaphis*

1) PD, Culture Collection, Plant Protection Service, Wageningen, the Netherlands
2) ATCC, American Type Culture Collection, Rockville, USA
3) NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK
4) RVCL, Royal Veterinary College, London, UK, courtesy A. Wood
5) WADA, Western Australian Department of Agriculture, South Perth, Australia, courtesy N. Buller
6) DAR, Department of Agricultural Research, Rydalmere, New South Wales, Australia, courtesy P. Fahy
7) IBSBF, culture collection of Phytobacteriology Section, Instituto Biologico, Sao Paulo, Brazil
8) CN, The Wellcome Bacterial Collection, Langley Court, Beckenham Kent, UK, courtesy S. Thorley
9) LMG, culture collection Laboratorium Microbiologie, Gent, Belgium, courtesy K. Kersters
10) CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France
11) MAFF, National Institute of Agrobacterial Resources, Dept. Genetic Resources II, Yatabe, Japan
12) ICMP, International Collection of Micro-organisms from Plants, Plant Diseases Division, Auckland, New Zealand
13) WCS, Phytopathology Laboratory 'Willie Commelin Scholten', Baarn, the Netherlands.
14) PCM, Polish Collection of Microorganisms, Wroclaw, Poland
15) CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia

TABLE 15. Reaction pattern of 21 non-identified *Pseudomonas* strains and *P. fluorescens* strains from *Iris* mentioned in Table 14.

	strains in fatty acid *P. fluorescens* cluster															outliers in fatty acid analysis						
	PD-number																					
Test	1246	1270	1276	1566	1592	1599	1715	1760	1762	1763	1771	1836	1054	212	*Iris* strains	123	1534	1538	1586	1247	1567	1752
Pyoverdin production	+	+	+	d	+	-	+	+	-[d]	-[d]	+	+	+	+	+	+	-[a]	+	-	-	+	+
Oxidase reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
Levan from sucrose	-	-	-	-	-	+	-	+	+	-	-	-	-	-	+	-	+	-	+	-	-	+
Gelatin hydrolysis	+	-	w	+	+	w	-	+	+	+	-	+	-	-	+	-	+	-	+	-	-	+
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lecithinase	++	-	-	+	+	+	-	+	+	-	-	+	-	-	-	-	++	-	+	+	-	+
Lipase (Tween 80)	++	-	-	+	+	-	-	-	+	+	+	+	-	+	w	-	+	+	+	+	-	+
Growth at 4°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Growth at 41°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Denitrification	-	w	-	w	-	-	w	w	-	w	w	-	-	-	-	-	+	-	+	-	-	-
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	-	+	-	+	+	+	-
Utilization of																						
Trehalose	-	-	d	+	-	+	-	+	+	+	-	-	-	d	d	-	+	-	-	-	d	-
2-Ketogluconate	+	d	-	-	-	w	-	d	-	+	-	-	-	-	-	-	+	-	-	-	-	-
m-Inositol	-	-	-	+	-	w	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+
Geraniol	-	-	+	-	-	-	-	d	-	-	-	-	-	-	-	-	-	d	-	-	d	-
L-Valine	-	d	d	-	d	-	-	d	d	-	d	+	-	-	-/w	-	+	-	-	-	-	-
β-Alanine	+	-	-	-	+	-	d	-	-	-	d	+	-	-	d	-	+	-	-	-	-	-
DL-Arginine	-	-	-	-	d	-	+	-	-	-	d	+	-	-	-	-	+	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-
Mannitol	+	-	-	+	+	-	+	+	d	d	+	-	+	-	d	-	+	-	-	+	-	-
L(+)Tartrate	-	+	d	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	-	-	-	-	-	+	+	d	+	+	-	-	-	d	+	-	-	-	-	-	+
Propionate	-	d	-	-	d	-	d	-	-	-	-	+	-	-	d	-	+	-	-	-	-	-
Saccharate	-	-	-	-	+	d	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
Propyleneglycol	-	-	d	+	+	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
D-Alanine	+	-	-	-	-	-	d	d	-	-	d	+	-	-	-	-	+	-	-	-	-	-
Ethanol	-	d	d	-	-	-	+	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-
DL-β-hydroxybutyrate	+	d	d	-	w	+	d	w	-	-	+	+	-	-	-	-	+	-	-	-	-	-
Sorbitol	-	-	-	-	-	+	-	+	w	d	+	-	-	-	-	-	+	-	-	-	-	-
Sucrose	-	d	-	-	d	-	+	-	-	-	d	-	-	d	d/w	-	+	-	-	-	-	-

+ = positive; ++ = strong positive; w = weak; d = doubtful; - = negative
a) = orange diffusable pigment produced

Distribution of plant-related characteristics: pectolytic and soft rot activity on potato disks and chicory heads

Table 14 shows that pectolytic activity was found to be absent from *P. aeruginosa* and *P. stutzeri* strains and the one strain studied from *P. chlororaphis, P. gladioli* pv. *gladioli, P. mendocina* and *P. syringae* pv. *syringae*. In all other taxonomic groups, however, pectolytic activity at pH 5.5 was present in one or more strains (Table 14). It was prevalent in *P. fluorescens* biovar 1 and 2 and *P. viridiflava* strains. Some strains showed pectinolysis or stronger pectinolysis at pH 8.

From the 74 strains which were pectolytic only 49% could produce soft rot of potato disks and 43% was pathogenic for chicory. Thus there was a close correlation between soft rot and pathogenicity on chicory. Twenty seven percent had some necrotizing effect on potato tissue and 5% had so on chicory. The pathotype strain of *P. marginalis* pv. *marginalis*, which we received from three different collections showed pectolytic activity only.

P. syringae and *P. cichorii* were not pectolytic but had a necrotizing effect on potato tissue. Four strains of *P. aeruginosa* also showed this behaviour on potato, two others caused necrosis on chicory only (Fig. 7). *P. aeruginosa* could be reisolated from the blackish necrotic tissue.

Most of the *P. fluorescens* biovar 2 strains, including those received as *P. marginalis* were pectolytic, caused soft rot and were pathogenic for chicory. Such strains, however, were also found in other taxonomic groups and non-pectolytic and/or soft rot/pathogenicity negative strains were found in biovar 2, too (Table 14).

P. fluorescens strains pathogenic to Iris 'Apollo'.

Nine *P. fluorescens* strains (PD 835-843) were isolated from *Iris* plants showing bacterial leaf stripe. The symptoms were longitudinal, irregular, glassy, brown to black stripes on leaves. These stripes could become up to 10 cm in length and the centre whitish, while the rest of the leaf yellowed (Fig. 8). The *Iris* strains were all non-pectolytic and showed no activity on potato or chicory. Upon inoculation of 5 of these strains in *Iris* 'Apollo' they caused similar symptoms as obtained in the field (Fig. 9).

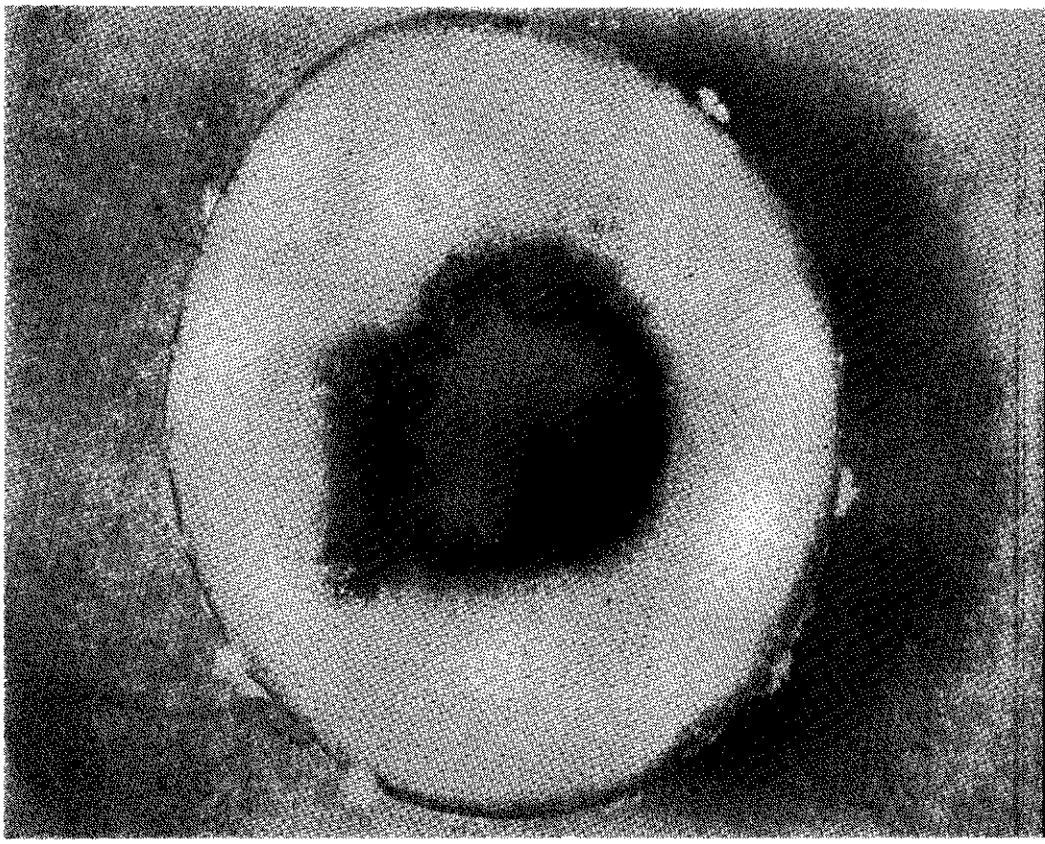

Fig. 7. Brownish-black necrotic tissue and green slimy growth caused by *Pseudomonas aeruginosa* PD 1531 from a sheep with green wool, one week after inoculation of a potato disk.

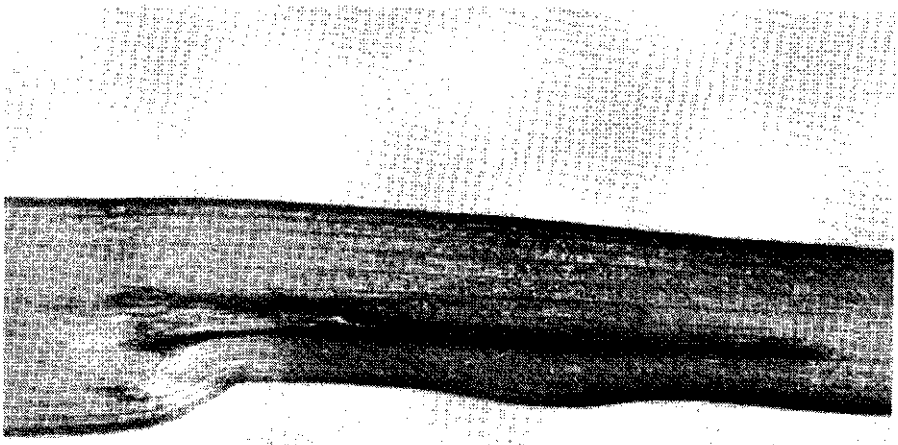

Fig. 8. *Iris* 'Blue Magic' showing symptoms of bacterial leaf stripe, caused by a non-pectolytic and soft rot negative strain of *P. fluorescens*. Natural infection, glassy to brown, longitudinal leaf spot with a white necrotic centre, surrounded by yellowing tissue.

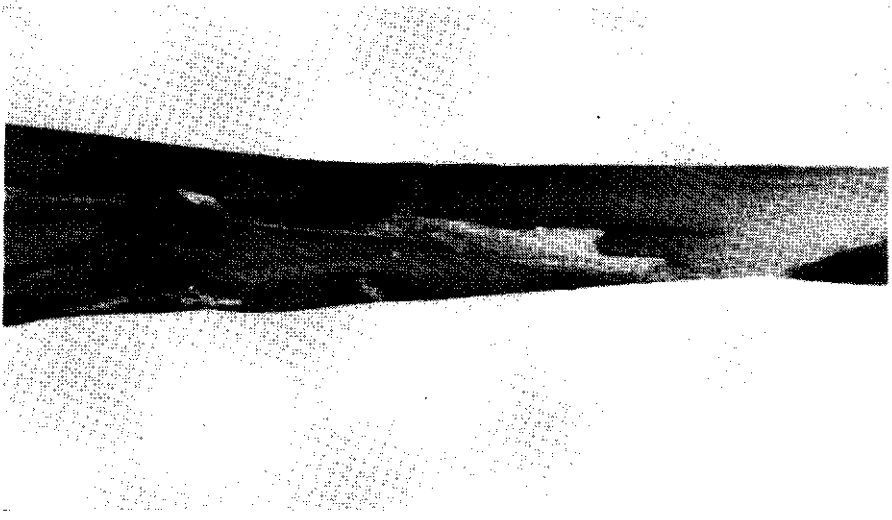

Fig. 9. *Iris* 'Apollo' showing symptoms of bacterial leaf stripe several weeks after artificial inoculation with *P. fluorescens* strain PD 841 isolated from naturally infected *Iris* spec. Symptoms similar to those in Fig. 8.

Classification of strains using fatty acid analysis

In Table 16 the mean percentage of the different fatty acids which were found in the taxonomic groups of Table 14 are presented. In all groups the acids 16:0, 16:1 cis 9 and 18:1 cis 11 counted for more than 70% of the total amount. Some fatty acids were only found in trace amounts in some strains: 10:0 and 13:0 3OH in *P. aeruginosa;* 15:0 in *P. aeruginosa, P. fluorescens* and *P. stutzeri;* 18:0 in all groups except *P. stutzeri;* 12:1 3OH in *P. aeruginosa* and *P. fluorescens;* 17:0 iso in *P. syringae* and *P. stutzeri* and 17:1 in *P. stutzeri.*

Table 16 shows that the non-fluorescent *P. gladioli* pv. *gladioli* is quite different from all other groups. Moreover it contained small amounts of the hydroxy acids 14:0 3OH (5%), 16:1 2OH (1%), 16:0 2OH (1%), 16:1 3OH (4%) and 18:1 2OH (2%). Also *P. aeruginosa, P. stutzeri* and the *P. syringae/viridiflava/cichorii* group differ significant from each other and the remaining groups. *P. fluorescens* and related groups (*P. aureofaciens, P. chlororaphis, P. putida),* however are very similar and show no significant differences.

TABLE 16. Whole cell fatty acid patterns of *Pseudomonas* spp. used in this study

Fatty acid	*P. gladioli* pv. *gladioli* (1)[a]	*P. stutzeri/mendocina* (4)	*P. aeruginosa* (25)	*P. cichorii/syringae/viridiflava* (12)	*P. fluorescens* (87)[b]	*P. fluorescens*-1 (13)	*P. fluorescens*-2 (38)	*P. fluorescens*-3 (3)	*P. fluorescens*-5 (18)	*P. aureofaciens/chlororaphis* (4)	*P. putida* (11)
saturated											
12:0	-	8	3	5	3	3	3	3	2	2	3
14:0	4[c]	1	1	-	t	t	1	t	t	t	t
16:0	27	19	25	27	32	32	33	30	32	32	30
18:0	1	-	1	2	1	1	1	1	1	1	1
unsaturated											
16:1 cis 9	15	25	14	36	29	28	29	31	28	28	25
18:1 cis 11	27	38	40	21	16	17	15	17	15	12	18
hydroxy											
10:0 3OH	-	4	5	3	4	4	4	3	5	6	5
12:0 2OH	-	-	4	3	4	4	4	5	5	5	4
12:0 3OH	-	4	5	4	5	5	5	5	5	6	5
cyclo											
17:0 cy	7	1	1	t	6	7	6	5	6	7	8
19:0 cy	5	t	1	-	t	t	t	t	t	-	t

a) = number of strains; b) deviating strains excluded; c) percentage; t = trace

When performing principal component analysis (PCA) with fatty acid patterns from all 164 strains four clusters were obtained (Fig. 10). Cluster A was a tight cluster comprising all *P. aeruginosa* strains, except PD 1358 which was slightly deviating and PD 277 which was very deviating. PD 277 was characterized by a much lower content of 16:0 (12%), the presence of 20:1 trans 11 (0.4%) and a very high percentage of 18:1 cis 11 (60%). Phenotypically, this strain behaved as a normal *P. aeruginosa* strain. Cluster B contained strains of *P. syringae* pv. *syringae* and the allied species *P. cichorii* and *P. viridiflava*. The only deviating strain was PD 1752, a *P. syringae* like organism (see table 15) from *Pachypodium lameri*. In cluster C *P. stutzeri* strains and the *P. mendocina* type strain were found. Cluster B, in fact a 'supercluster', contained most of the *P. fluorescens* and related strains *(P. aureofaciens, P. chlororaphis, P. putida* and *P. tolaasii)*. It included all strains received as *P. marginalis* except the pathotype strain of *P. marginalis* pv. *alfalfae*, PD 1556. This strain was found to be non pectolytic and non pathogenic. In the supercluster there were no separate clusters discriminating biovars of *P. fluorescens*, the other related species or strains received as *P. marginalis*. Many "P. marginalis" and other *P. fluorescens* biovar 2 strains were found near the centre, but others scattered through the cluster.

Seven of the nine strains isolated from *Iris* with bacterial leaf stripe symptoms formed a subcluster at the top of cluster D (Fig. 10). Strains which were found to be isolated from the clusters are also represented in Fig. 10. Most deviating was PD 1586, a non-fluorescent pseudomonad received as *P. chlororaphis*. Some of the isolated strains (PD 1247, 1534, 1567, 1586 and 1752) were also abarrant in biochemical tests (Table 15). Other biochemically abarrant strains from Table 15 all fitted well in the *P. fluorescens* super-cluster.

When performing cluster analysis with fatty acid patterns of all 164 strains essentially the same taxonomic structure and classification was obtained as with PCA (Fig. 11). Again there was a tight cluster (no. IX) of *P. aeruginosa*, cluster I and II of the *P. syringae, viridiflava, cichorii* and no. VIII of the *P. mendocina - stutzeri* group and a supercluster (clusters III to VII) of *P. fluorescens* and related species. Biovars of *P. fluorescens*, soft rot pseudomonads or strains received as *P. marginalis* could not be discriminated.

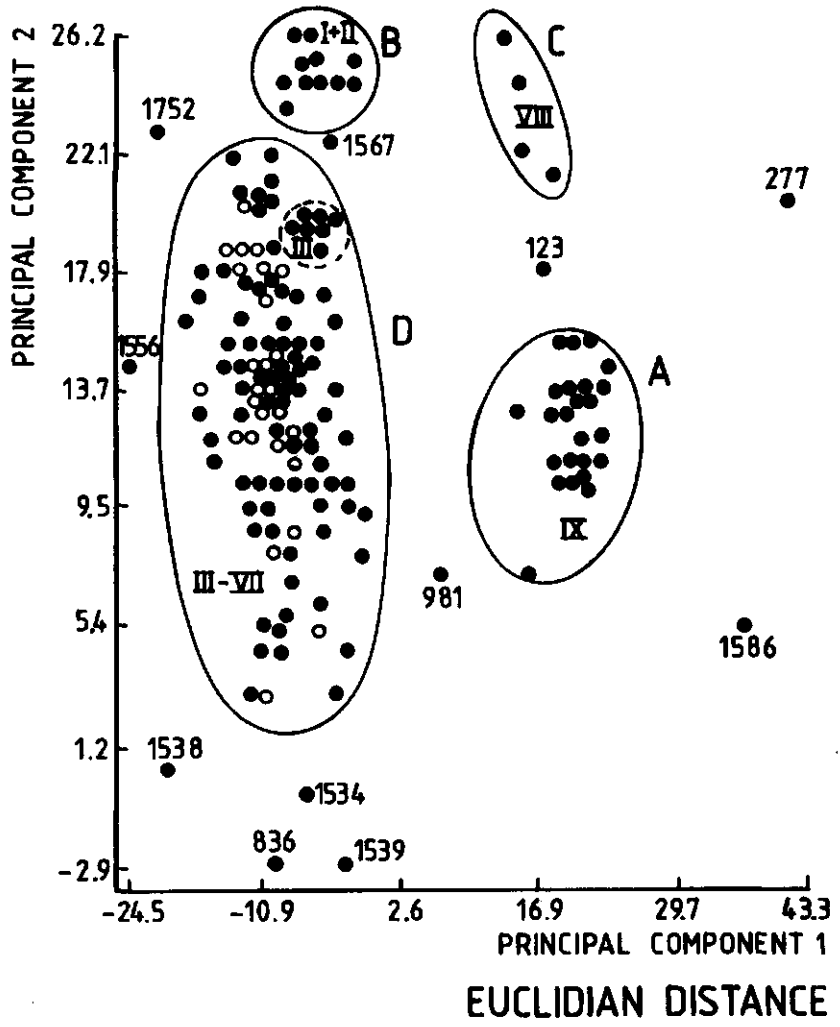

Fig. 10. Two-dimensional plot of principal component analysis of 164 *Pseudomonas* strains showing subdivision into four clusters. Cluster A corresponds to *P. aeruginosa* strains, cluster B to *P. viridiflava, P. cichorii* and *P. syringae* strains, cluster C to *P. stutzeri* and *P. mendocina* strains, cluster D to strains received and/or phenotypically identified as *P. fluorescens* biovars 1, 2, 3 and 5. *P. aureofaciens,* as *P. chlororaphis,* as *P. marginalis* and *P. tolaasii.* For individual strain numbers see Table 14. Roman numbers correspond to clusters from Fig. 5. o = strains received as *P. marginalis* and/or identified *P. fluorescens* biovar 2. ● = strains other than o.

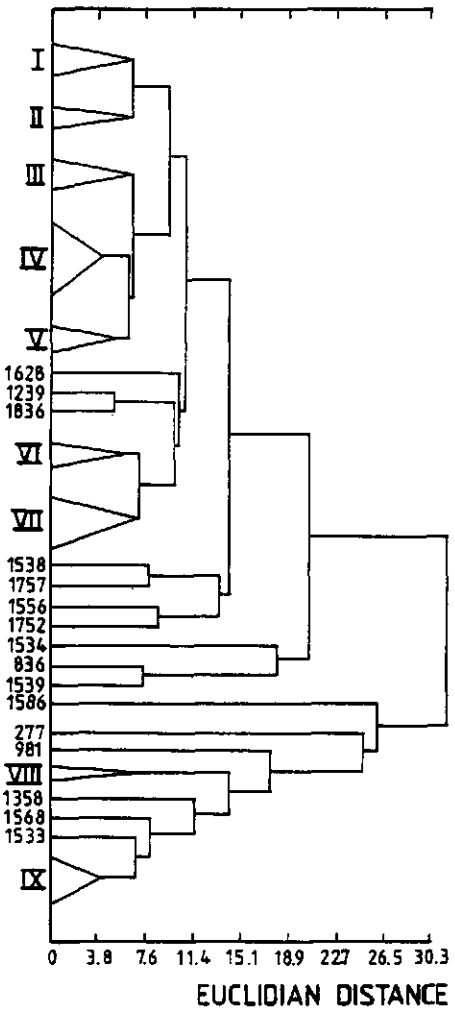

Fig. 11. Dendrogram showing relationships of 164 *Pseudomonas* strains, obtained in cluster analysis. Cluster I and II, *P. cichorii, P. syringae* and *P. viridiflava* strains and PD 1591 and PD 1326; cluster III-VII strains received and/or identified as *P. aureofaciens, P. chlororaphis, P. fluorescens, P. marginalis* and *P. tolaasii;* cluster VIII, *P. stutzeri* and *P. mendocina* strains and PD 123; cluster IX, *P. aeruginosa* strains. For individual strain numbers see Table 14.

Discussion

Fatty acid patterns of different *Pseudomonas* species determined in this study were remarkably similar to those reported by others (Chen & Chen, 1981; Conrad et al., 1981, Ikemoto et al., 1978; Moss & Dees, 1976; Oyaizu & Komogata, 1983). With our growth conditions, extraction method and chromatograph we did not detect 19:0 (found sometimes to be present at 1-2%) or 14:0 iso, 16:0 iso (Conrad et al., 1981), the percentages 17:0 cy and 19:0 cy were usually lower, 12:0 3OH, 16:1 and 18:1 usually higher than reported by other authors. The acids 17:0 cy and 19:0 cy are related to 16:1 and 18:1 in this way that in physiologically aging cells 16:1 and 18:1 are converted to 17:0 cy and 19:0 cy respectively (Rose, 1989). We used actively growing cells (late log phase).

Using fatty acid patterns of 164 *Pseudomonas* strains in Principal Component Analysis (PCA) most strains were found in four clusters. One (super)cluster comprised the majority of strains identified as *P. aureofaciens, P. chlororaphis, P. fluorescens, P. marginalis* and *P. tolaasii.* In the second cluster the oxidase-negative *P. syringae, P. viridiflava* and the related oxidase-positive, soft rot negative pathogen *P. cichorii* were found. This clustering is in accordance with DNA homology studies (Pecknold & Grogan, 1973). The third cluster contained non-fluorescent *P. mendocina* and *P. stutzeri* strains. The close relationship of the latter two species was also noted by Palleroni & Doudoroff (1972), Chen & Chen (1981) and Moss & Dees (1976). The fourth tight cluster contained all but one of the *P. aeruginosa* strains. The deviating strain showed a high amount of 18:1, raised levels of this acid were found to be characteristic for strains resistant to chloramphenicol or polymyxin B (Anderes et al., 1971; Conrad & Gilleland, 1981).

The PCA analysis of fatty acid patterns did not reveal separate clusters comprising strains received or identified as *P. marginalis* or as fluorescent pseudomonads soft rot. The oxidase positive soft rot strains and *P.marginalis* were present in the *P. fluorescens* super cluster and the oxidase-negative soft rot strains in the *P. syringae/viridiflava/cichorii* cluster. In relation to these findings Lelliott et al. (1966) almost prophetically stated in their study on fluorescent pathogenic pseudomonads: '*P. marginalis* and *P. pastinaceae* are possibly synonyms, but the boundery between these species, the other soft rotting organisms of group IV, and the *P. fluorescens* complex is not clear, and it is at least possible that these pathogens differ from the complex of saprophytic, fluorescent Pseudomonads which inhabit soil, water and plant surfaces only in their ability to produce (or to produce in vivo) the

enzymes responsible for soft rots. *P. tolaasii* may also be closely related to the *P. fluorescens* complex'.

Our study leads to conclusions which are similar or very close to the guesses of Lelliott et al. (1966), as will be discussed below. The ability to cause soft rot appears to be present in most of the biovars of *P. fluorescens*, in phenotypic intermediates of these biovars, in *P. putida*, *P. aureofaciens*, *P. tolaasii*, in strains received as *P. marginalis* and several strains which could not be identified by phenotypic tests. This widespread occurrence of the soft rot property in fluorescent pseudomonads was also found by Brocklehurst & Lund, 1981; Campbell et al., 1986; Liao, 1987; Samson, 1981; Sands & Hankin, 1975; Vantomme et al., 1989; Wang & Kelman, 1987; Wells & Liao, 1987. For separate strains belonging to biovars of *P. fluorescens* other than biotype 2 it was reported by e.g. Calzolari & Bazzi, 1985; Malathrakis & Goumas, 1987; Sellwood et al., 1981; Surico & Iacobellis, 1978. The occurrence of sot rot activity in so many diverse fluorescent pseudomonads fatally weakens the case for preserving a species designation for soft rot strains which conform to biovar 2 of *P. fluorescens* (= *P. marginalis* sensu stricto). It must be said, however, that many soft rot strains are found to be more or less similar to biovar 2 and many produce a pink to brownish diffusable pigment (this study, Brocklehurst & Lund, 1981; Cuppels & Kelman, 1980). But in fatty acid analysis these strains did not form a separate group and were found scattered in the *P. fluorescens* supercluster. Moreover non-pathogenic biovar 2 strains are found, indistinguishable from the soft rot strains and pink/brown pigment production was also found in biovar 5 soft rot strains.

Upon phenotypic identification many of our strains did not fit or did not fit well into the known taxonomic entities, i.e. fluorescent *Pseudomonas* species or biovars of *P. fluorescens*. Difficulties in classifying fluorescent pseudomonads from other sources and origins into the known taxonomic entities were reported by Brocklehurst & Lund, 1981; Hildebrand, 1989; Hildebrand et al, 1984; Molin & Ternstrom, 1986; Van Outryve et al, 1989; Samson, 1982; Sands & Hankin, 1975; Sands & Rovira, 1971, Sneath et al., 1981 and Vantomme et al., 1989. This difficulty in identification of strains could be due to several reasons, most of them also mentioned by Sands & Rovira (1971): a) deviations or errors due to the use of different chemicals and media or to difficulties in interpretation of weak and doubtful reactions; b) difference in strains which were freshly isolated and those which have been in culture collections for a long time; c) strains obtained in enrichment cultures, as many of those characterized by Stanier et al. (1966), may be more uniform than those randomly isolated. This is perhaps demonstrated by our strains from diseased *Iris* (the *Iris* plant as enrichment medium), most of these strains were very uniform in all tests; d) strains

from diverse habitats and/or geographic origins show more variation than the present schemes (Palleroni, 1984) allow.

The growing body of evidence strongly suggests that reason d) i.e. more variation present in *P. fluorescens* and related species than initially expected, is most important. In fatty acid analysis most of the phenotypically intermediate strains or those showing unknown patterns were found in the *P. fluorescens* supercluster together with *P. fluorescens, P. aureofaciens, P. chlororaphis, P. putida* and *P. tolaasii*. Formerly *P. aureofaciens* and *P. chlororaphis* were described as biotypes of *P. fluorescens* and recently combined by Johnson & Palleroni (1989) into one species, viz. *P. chlororaphis*. *P. putida* was formerly named *Bacillus fluorescens non-liquefaciens* and was also found to be closely related to *P. fluorescens* by Hildebrand et al., 1984 and Sneath et al., 1981. The one strain of *P. tolaasii* we tested, showed a phenotypic pattern of *P. fluorescens* biovar 5. Within the *P. fluorescens* supercluster there were no clear clusters which corresponded to current taxonomic entities. The conclusion seems inescapable that there is a large, but circumscribed complex continuum of fluorescent, oxidase positive pseudomonads, called *P. fluorescens*. From the above mentioned facts it must be clear that it is not justifiable to lump all the diverse oxidase positive, fluorescent soft rot pseudomonads, all in the *P. fluorescens* supercluster, in a separate, completely artificial species *P. marginalis (P. marginalis* sensu lato).

The conclusion is that the use of the specific epithet *Pseudomonas marginalis* (sensu stricto and sensu lato) has to be abandoned. A pathovar rank for the opportunistic soft rot pseudomonads, such as *P. fluorescens* pv. *marginalis* is not desirable. The term pathovar should be confined to strains of true plant pathogens showing a **distinctive** pathogenicity to one or more hosts (Dye et al, 1980). Moreover not much is known about host range of soft rot strains and opportunistic *P. fluorescens* strains not showing soft rot, like our strains from *Iris* and those described by Hevesi et al. (1978). The status of the two pathovars (pv. *pastinacae* and pv. *alfalfae)* of *P. marginalis* is not clear. The strains we tested were not causing soft rot on potato or chicory. It is not known if this is due to absence or loss of pathogenicity or difference in host range. It is suggested here that oxidase positive, fluorescent soft rot pseudomonads should be distinguished in future as follows:

- *P. fluorescens* (pectolytic, soft rot strain), or if one also wants a phenotypic characterization:
- *P. fluorescens* (phenotype biovar 2, pectolytic, soft rot strain) or
- *P. fluorescens* (phenotype *P. putida*, pectolytic, soft rot strain).

Oxidase negative, fluorescent soft rot pseudomonads can still be named *P. viridiflava*. *P. viridiflava* appears to be homogeneous, but strains may easily loose pathogenicity (Lukezic et al., 1983; Lund 1983; Vantomme et al., 1989). Perhaps other characteristics which determine opportunistic pathogenicity in *P. fluorescens* such as production of wetting agents (Hildebrand, 1989), proteolytic enzymes (Wang & Kelman, 1987) or other, still unknown factors (*Iris* strains) could be included in the above mentioned naming.

In sharp contrast to the heterogeneity of *P. fluorescens* is the homogeneity of *P. aeruginosa*, both in phenotypic reactions and fatty acid patterns. This homogeneity has also been observed by others (p.e. Champion et al., 1980; Jessen, 1965; Liston et al., 1963; Stanier et al., 1966). There were no obvious differences between strains from plants, animals or man. In our study we could not confirm an opportunistic (soft rotting) plant pathogenicity of *P. auruginosa* as reported by Cho et al. (1975), Cother et al. (1976) and Elrod & Braun (1942). Only some strains, from animal, man and plants caused occasionally necrosis of potato or chicory tissue. Even in the more recent study of Cother et al. (1976) only 3 out of 300 onions showed symptoms after inoculation of a *P. aeruginosa* strain isolated from brown onion scales. *P. aeruginosa* therefore should be regarded as a widespread saprophyte on plants and in soil (Cho et al., 1975; Green et al., 1974) with an opportunistic pathogenicity towards animals and man. It may under certain circumstances multiply and exert some necrotizing action (by unknown factors) in plant tissue.

P. fluorescens strains causing bacterial leaf stripe of *Iris* have not been described before. Bacterial blight symptoms, which are different from bacterial leaf stripe, are caused by *Xanthomonas campestris* pv. *tardicrescens* in the USA (Burkholder, 1937). A leaf scorch and root rot said to be caused by a fluorescent pseudomonad was reported by Bald (1971). The pathogenicity factors of *Iris* strains are still unknown, since they were not pectolytic and did not show ice nucleation activity.

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Es giebt ein vollendetes organisches Leben im unsichtbar kleinen Raume, welches die Grösse des Grossen in der Natur unabsehbar erhebt.

Christian Gottfried Ehrenberg

III. GENERAL DISCUSSION

In this study the taxonomy of some plant pathogenic bacteria, belonging to the genus *Pseudomonas*, has been investigated. Both conventional physiological, biochemical and pathological methods were used as well as a relatively new finger-print technique, viz. automated whole cell fatty acid analysis (FAA). I found FAA to be of great value for defining and identifying plant pathogenic pseudomonads at and below species level. The Microbial Identification System (MIS) of Microbial ID inc., Newark, DE, USA allowed acquisition of very reproducible fatty acid profiles through standardized growth conditions, harvesting, extraction, gaschromatographic processing and data handling.

It should be realized that some of the differences found in fatty acid composition may be caused by difference in growth rate between organisms. This difference in growth rate, apart from being dictated directly by the genome, may also be due to differences in temperature sensitivity or nutritional demands. This implies that by changing temperature or growth medium some differences may disappear, others may be revealed. The sensitivity to these kind of changes appears to be different between organisms (Rose, 1989). Standardization has also been found necessary when other structural elements such as proteins, cell wall polymers or lipids are used for classification purposes (Ellwood & Tempest, 1972, Goodfellow & Minnekin, 1985).

The extraction method followed in the MIS protocol is a compromise between simplicity and rapidity on one hand and optimal recovery on the other. Furthermore the type of column may influence the visualization of differences in fatty acid composition (Horning et al., 1969, Moss et al., 1974). The saponification and methylation method and the column used by the MIS guarantee a good recovery and separation of (isomers of) almost all fatty acids present in bacteria (Miller, 1982; Sasser, 1990). Some technical perfection could possibly be achieved by miniaturizing harvest-extraction steps. At present 40 mg wet weight of cells is used to obtain c. 0.5 ml extract. From this extract, however, 2 µl is injected while only 0.02 µl actually enters the column.

Where studied, FAA yields taxonomic patterns which are generally in good congruence with those of genomic methods such as DNA-DNA or DNA-r(ibosomal) RNA homology studies or Restriction Fragment Length Polymorphism Analysis, RFLP. In comparison with RFLP, FAA is less sensitive. With RFLP strain specificity can be obtained, which is important in ecological studies (Cook et al., 1990; Kuykendall et al., 1988; Oyaizu & Komagata, 1983; Sasser & Smith, 1987; this study, chapter II.3).

In the past it was found that oxidase negative, fluorescent, plant pathogenic pseudomonads could not easily be distinguished in the laboratory. Distinction was mainly based on pathogenicity, host range and symptomatology. Therefore most of these species were clustered in one species, *P. syringae*, by Doudoroff et Palleroni (1976). Because the pathogenic individuality of the former (nomen)species was lost, Dye et al. (1980) revived most of them as pathogenic varieties (pathovars) of *P. syringae*. Unfortunately, in this case both real pathovars, i.e. organisms differing from *P. syringae* in host range and symptomatology only, and organisms deserving a higher taxonomic rank were equalized in a special purpose classification. With special purpose classification is meant a classification constructed for a limited purpose only, in the sense of Gilmour (1937, 1961). What happened was that nomenspecies became nomenpathovars.

An example of the limitations of the pathovar system is described in this study for *P. syringae* pv. *savastanoi*, formerly *P. savastanoi*, causing wartlike excrescences or galls on *Oleaceae* and *Nerium oleander*. This bacterium differs biochemically, serologically and pathologically from *P. syringae*, and therefore was given a subspecies rank. Moreover it contains three pathovars, viz. pv. *oleae*, pv. *nerii* and pv. *fraxini*, differing in host range, plant hormone production and pathogenicity (chapter II.1). The differentiation into pathovars could be confirmed by FAA (chapter II.2) and recently also by genome digestion patterns (Mugnai et al., 1991).

The positive results in discriminating pathogenic varieties of *P. syringae* subsp. *savastanoi* by FAA stimulated a study on an important non-fluorescent plant pathogen viz. *P. solanacearum*. This bacterium causes bacterial wilt of many different plants and belongs to the rRNA group II as defined by Palleroni et al., 1973. It has a world wide distribution and biochemical as well as pathogenic varieties (called races) have been described. With FAA the biochemical varieties could not be discriminated. Apparentlty the difference in ability to oxidize a few carbon compounds does not evoke changes in the cell membrane, where most fatty acids are located. (Russell, 1989). As with pathovars of *P. syringae* subsp. *savastanoi*, pathogenic varieties of *P. solanacearum* could be distinguished by FAA (Chapter II.3). Race 1 of *P. solanacearum* which has a

very wide host range, was found to be very heterogeneous in biochemical tests and FAA. Race 3, which has a very narrow host range (potato, tomato, some solanaceous weeds) was found to be very homogeneous. This is a peculiar, unexplained phenomenon, also occurring in other bacterial groups. *P. aeruginosa*, for example is very homogeneous, in sharp contrast to *P. fluorescens* (Stanier et al., 1966; this study, chapter II. 3 and 4). *P. mendocina* is homogeneous, *P. stutzeri* very heterogeneous (Stanier et al., 1966). In 5S rRNA sequence and DNA-DNA homology studies *Escherichia coli* was very homogeneous, *Bacillus circulans* very heterogeneous (Sneath, 1989). The role of the host or the habitat does not seem to be very important as *P. solanacearum* race 1 and 3 both occur in potato and tomato. *P. aeruginosa* and *P. fluorescens* occur in many, similar habitats.

When studying *P. solanacearum* it also proved to be possible to identify the pathogenic variety (race) of strains which had lost pathogenicity. This is normally impossible, except in certain cases with RFLP analysis (Cook et al., 1990). Similarly non-nodulating strains of the root-nodulating, nitrogen-fixating *Frankia* species (*Actinomycetacae*) could be allocated by FAA to their genus, which is impossible by other methods (Mirza et al., 1991). *P. solanacearum* strains were also studied with FAA in their relation to other species of rRNA group II. A similar taxonomic pattern as found in DNA-DNA homology studies (Palleroni & al., 1973) was found. Also in this way FAA appears to be a powerful, rapid and reliable tool to study affinity between bacterial taxa.

After FAA was successfully used in the taxonomy of *P. syringae* subsp. *savastanoi* and *P. solanacearum*, the very complex group of the fluorescent, oxidase positive pseudomonads, causing soft rot of plants, was tackled. These bacteria, named *P. marginalis* or *P. fluorescens* appear biochemically indistinguishable from biovars 1, 2, 3 and 5 of *P. fluorescens*, are intermediates of these biovars or are similar to *P. chlororaphis* or *P. putida*, all in rRNA group I. The pathogenicity of the soft rot strains is of a secondary type, i.e. they can cause disease under adverse conditions for the plant and only following injury. This type of pathogenicity is apparently fundamentally different from those of primary pathogens as it is not reflected in biochemical behaviour, protein or fatty acid composition (Van Outryve & al., 1989; Vantomme & al., 1989; this study, chapter II.4). The soft rot pseudomonads formed a large FAA cluster with saprophytic *P. fluorescens*, *P. chlororaphis* and *P. putida* strains. Therefore I suggested a pragmatic special purpose classification for soft rot strains (p.e. *P. fluorescens*, pectolytic soft rot strain), saving information for the pathologist and yielding mono- or oligothetic groups within *P. fluorescens*. Moreover it was found that there was no clear distinction between biovars of *P. fluorescens*,

P. chlororaphis and *P. putida*. When studying a large number of newly isolated strains, many biochemically intermediates were found, violating the biovar system (Palleroni, 1984). Because this phenomenon was also noted by others, all soft rot strains were placed in this study in *P. fluorescens*. Thus there is a need for a thorough reevaluation of the ecological significance, the biological basis and the necessity of biochemical subdivision in the *P. fluorescens* cluster. It should be remembered that strains from a similar host or habitat, selected and isolated by enrichment may yield strains which form relatively homogeneous nests in the large *P. fluorescens* cluster (Stanier & al., 1966), as was found with non-pectolytic, non-soft rot strains causing leaf stripe of *Iris* (this study, chapter II.4).

In the study on the oxidase positive fluorescent, soft rot pseudomonads also strains of non-pectolytic, non-soft rot *P. fluorescens*, causing leaf stripe of *Iris* and of the opportunistic pathogen of men and animals, *P. aeruginosa* were included. From the *Iris* strains the pathogenicity factor has not been elucidated and this needs further research. *P. aeruginosa* has been described as a pathogen of plants on several occasions (Cother, 1976; Elrod & Brown, 1942). Its plant pathogenicity was not well established, however. We found that only in some cases, for unknown reasons, both strains from animal or plant origin exerted some necrotizing effect on potato or chicory tissue. The conclusion is that *P. aeruginosa* is not a plant pathogen, but that this bacterium may sometimes be found and multiply on or in plant tissue in regions with a warm climate.

From this study it is concluded that the most useful, natural classification of plant pathogenic pseudomonads will be obtained by studying them from as many angles as possible. With 'natural classification' is meant a classification in the sense of Gilmour (1937, 1961, also see Sneath, 1988), based on overall similarity of a large number of characters, having a large information content. A combination of methods such as (numerical analysis of) phenotypic determinations, pathogenicity and host range studies, FAA, protein profiling and nucleic acid homology and sequence studies, will lead to a better understanding of the interesting and economically important group of plant pathogenic pseudomonads. Using or absolutizing only one of these methods will lead to reductionistic, insufficient and/or unworkable classifications.

Finally this study revealed that pathogenicity of primary pathogens (where a lot of genetic information is involved concerning chemotaxis, recognition, contact, ingress and establishment in the host), should not be regarded as just one phenotypic character. In this respect still a lot of information is lacking, especially at the infraspecific level.

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SUMMARY

In this thesis some members of the plant pathogenic bacterial genus *Pseudomonas* have been studied. Conventional morphological, biochemical, physiological and pathogenitcity tests as well as a 'finger-print' technique, viz. automated whole cell fatty acid analysis, were used. The taxonomy of the plant pathogenic *Pseudomonas* bacteria is in many cases unsettled. Not in the last place this is because plant pathogenicity is difficult to assess and its value in taxonomy is differently estimated. The genus *Pseudomonas* has been subdivided on the basis of DNA-r(ibosomal) RNA homology studies into four rRNA groups. Fluorescent plant pathogens are found in group I, non-fluorescent species in group II (Chapter I.2.).

Whole cell fatty acid analysis has been found very useful in the classification of phytopathogenic bacteria. Especially the development of the Microbial Identification System (MIS) of Microbial ID. Inc. Newark, USA enables rapid and reliable determination of fatty acid patterns and has enhanced its use in bacterial taxonomy (Chapter I.3.). The MIS has been used in all my studies on fatty acids of plant pathogens.

At first a bacterium was studied which was well known in our laboratory, viz. the fluorescent *P. syringae* subsp. *savastanoi*, causing excrescences on *Oleaceae* and *Nerium oleander*. Based on pathogenicity, host range and plant hormone production three pathogenic varieties of this bacterium could be distinguished (Chapter II.1.). With fatty acid analysis (FAA), the three pathovars could also be distinguished, leading to the notion that pathogenicity of primary plant pathogens is not just one phenotypic factor, but it is also reflected in the bacterial membranes, where most fatty acids are present (Chapter II.2.).

Subsequently the important non-fluorescent plant pathogen *P. solanacearum* was investigated. From this bacterium, which causes a devastating vascular disease on many different food crop plants, both biochemical and pathogenic varieties have been described. The occurrence of biochemical varieties could not be confirmed by FAA. Apparently differences in the ability to metabolize a few carbon compounds have no effect on fatty acid composition. As was the case with *P.s.* subsp. *savastanoi*, pathogenic varieties of *P. solanacearum* could be discriminated by FAA. Fatty acid patters of *P. solanacearum* were also studied in relation to those of other members of rRNA-group II, such as *P. cepacia, P. gladioli, P. caryophylli* and *P. pickettii*. The taxonomic patterns found were in good congruence with those determined in

DNA-DNA homology studies by other authors. This once more confirms that FAA is a powerful additional tool in the classification of bacteria (Chapter II.3.).

Finally the very complex group of the fluorescent, oxidase positive soft rot *Pseudomonas* bacteria was studied. These bacteria are opportunistic plant pathogens, especially important in post harvest situations. They have been found to be biochemically indistinguishable from saprophytic pseudomonads such as *P. fluorescens* biovars, *P. putida* and *P. chlororaphis* (incl. *P. aureofaciens*). On the basis of their ability to hydrolyze pectin and to cause soft rot, they have been named *P. marginalis*. In this study soft rot strains were biochemically similar to biovars of *P. fluorescens* or intermediates of these biovars, unknown forms of *P. fluorescens*, or similar to *P. putida* and *P. chlororaphis*. With FAA, oxidase positive sof rot pseudomonads were all found in a heterogeneous super cluster with saprophytic strains biochemically identified as *P. fluorescens* biovars or intermediates, *P. chlororaphis* and *P. putida*. Therefore it is suggested to abandon the use of '*P. marginalis*' and to name oxidase positive fluorescent soft rot bacteria '*P. fluorescens*', with some additional information between brackets, e.g. *P. fluorescens* (pectolytic, soft rot strain).

P. aeruginosa strains from plants, animals and men were found in a very homogeneous cluster, well separated from the *P. fluorescens* supercluster. A supposed plant pathogenicity of *P. aeruginosa* could not be confirmed. This bacterium can multiply and cause some necrotic action only occasionally on plant material under special (unknown) circumstances. Certain non-pectolytic, non-soft rot strains of *P. fluorescens* are described which cause bacterial stripe symptoms on *Iris* sp. The pathogenicity factors of the *Iris* strains have not been substantiated (Chapter II.4.).

Fatty acid analysis has been shown to be a welcome and useful tool in elucidation of natural relations between plant pathogenic *Pseudomonas* bacteria. Fatty acid analysis in combination with other methods such as conventional phenotypic tests and DNA-and protein fingerprinting may lead to a better understanding of this interesting group of bacteria, not in the last place to achieve a better disease control (Chapter III).

SAMENVATTING

In dit proefschrift werden enkele vertegenwoordigers van het plantepathogene bacteriegeslacht *Pseudomonas* bestudeerd. Hiervoor werden conventionele morfologische, biochemische, fysiologische en pathogeniteits toetsen gebruikt alsmede een 'vingerafdruk' methode, te weten geautomatiseerde analyse van de totale vetzuursamenstelling van bacteriën, kortweg vetzuuranalyse. De taxonomie van de plantepathogene *Pseudomonas* bacteriën is in veel gevallen nog onduidelijk. Dit niet in de laatste plaats omdat pathogeniteit vaak moeilijk is vast te stellen, terwijl de waarde ervan in de taxonomie verschillend wordt ingeschat. Het geslacht *Pseudomonas* is ingedeeld, op basis van DNA-r(ibosomaal) RNA homologie studies, in vier rRNA groepen. Fluorescerende plantepathogenen worden gevonden in groep I, niet-fluorescerende soorten in groep II (Hoofdstuk I.2.).

Vetzuuranalyse is zeer bruikbaar gebleken bij de classificatie van (plantepathogene) bacteriën. Speciaal de ontwikkeling van het zogenaamde Microbial Identification System (MIS) van Microbial ID. Inc., Newark, V.S. heeft de snelle en betrouwbare bepaling van vetzuurpatronen mogelijk gemaakt en het gebruik ervan in de taxonomie gestimuleerd (Hoofdstuk I.3.). Het MIS werd toegepast bij al mijn onderzoeken omtrent vetzuren van plantepathogene bacteriën.

Eerst werd een bacterie bestudeerd die goed bekend was in ons laboratorium, namelijk de fluorescerende *P. syringae* subsp. *savastanoi*, welke woekeringen veroorzaakt bij *Oleaceae* en *Nerium oleander*. Op basis van pathogeniteit, waardplantenreeks en plantehormoon produktie konden drie pathogene variëteiten van deze bacterie worden onderscheiden (Hoofdstuk II.1.).
Deze pathogene variëteiten konden ook met behulp van vetzuuranalyse onderscheiden worden. Dit leidde tot het vermoeden dat pathogeniteit van primaire pathogenen niet slechts één fenotypische factor is, maar dat zij ook wordt weerspiegeld in de bacteriële membranen, waar de meeste vetzuren gevonden worden (Hoofdstuk II.2.).

Vervolgens werd de belangrijke niet-fluorescerende plantepathogene bacterie *Pseudomonas solanacearum* aan een onderzoek onderworpen.
Van deze bacterie, die een verwoestende vaatziekte veroorzaakt bij vele verschillende voedselplanten, zijn zowel biochemische als pathogene variëteiten beschreven. Het bestaan van biochemische variëteiten kon niet met vetzuuranalyse worden bevestigd. Blijkbaar heeft het verschil in mogelijkheid enkele koolstofbronnen te kunnen metaboliseren geen effect op de vetzuursamenstelling. Zoals ook bij *P.s.* subsp. *savastanoi* konden de pathogene variëteiten van *P. solanacearum* met vetzuuranalyse

worden onderscheiden. Vetzuurpatronen van *P. solanacearum* werden ook bestudeerd in relatie tot andere soorten behorend tot rRNA groep II zoals *P. cepacia, P. gladioli, P. caryophylli* en *P. pickettii.* Het taxonomische patroon wat gevonden werd, kwam goed overeen met het patroon vastgesteld in DNA-DNA homologie studies van andere auteurs. Dit bevestigt nog eens dat vetzuuranalyse een uiterst waardevolle additionele methodiek is bij de classificatie van bacteriën (Hoofdstuk II.3.).

Tenslotte werd de zeer complexe groep van de fluorescerende, oxidase positieve natrot *Pseudomonas* bacteriën bestudeerd. Deze bacteriën zijn opportunistische pathogenen, met name belangrijk onder bewaarcondities na de oogst. Zij bleken biochemisch niet te onderscheiden van saprofytische pseudomonaden zoals biovars van *P. fluorescens, P. putida* en *P. chlororaphis* (inclusief *P. aureofaciens*). Op basis van hun vermogen pectine te hydrolyseren en natrot te veroorzaken, werden zij *P. marginalis* genoemd. In de onderhavige studie bleken natrot stammen biochemisch gelijk aan biovars van *P. fluorescens,* of zij waren intermediairen van deze biovars, onbekende vormen van *P. fluorescens,* of gelijk aan *P. putida* of *P. chlororaphis.* Bij vetzuuranalyse werden alle oxidase positieve natrot pseudomonaden in een heterogene supercluster gevonden, samen met saprofytische stammen die biochemisch werden geïdentificeerd als *P. fluorescens* biovars of intermediairen, *P. chlororaphis* of *P. putida.* Daarom wordt voorgesteld het gebruik van '*P. marginalis*' niet langer te handhaven en de oxidase-positieve fluorescerende natrot bacteriën *P. fluorescens* te noemen, met enige toegevoegde informatie tussen haakjes, bijvoorbeeld *P. fluorescens* (pectolytische natrot stam).

P. aeruginosa stammen van plant, mens en dier werden in een zeer homogene cluster gevonden, die duidelijk gescheiden was van de *P. fluorescens* supercluster. Een veronderstelde plantepathogeniteit van *P. aeruginosa* kon niet worden bevestigd. Deze bacterie kan zich alleen incidenteel vermenigvuldigen en enige necrose veroorzaken in planteweefsel onder speciale (onbekende) omstandigheden.
Er werden zekere niet-pectolytische, niet-natrot veroorzakende stammen van *P. fluorescens* beschreven die bacteriële strepenziekte veroorzaken bij *Iris* soorten. De pathogeniteitsfactoren van de *Iris*-stammen konden niet worden vastgesteld (Hoofdstuk II. 4.).

Vetzuuranalyse is een welkome en bruikbare methode gebleken voor het ophelderen van natuurlijke relaties tussen plantepathogene *Pseudomonas* bacteriën. Vetzuuranalyse, in combinatie met andere methoden zoals conventionele fenotypische toetsen en DNA-en eiwit vingerafdrukmethoden, kunnen leiden tot een beter begrip van deze interessante groep van bacteriën, niet in de laatste plaats met het oog op een betere ziektebestrijding (Hoofdstuk III.).

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Curriculum Vitae

De auteur van dit proefschrift werd op 28 juni 1953 te Rijswijk (ZH) geboren. Na het HAVO diploma (1972) werd het diploma van de 3-jarige Rijks Middelbare Landbouwschool te Leeuwarden behaald (1974). De interim tussen RMLS en militaire dienst werd opgevuld met een half jaar onderzoek aan het Proefstation voor de Rundveehouderij te Wageningen naar de ruwvoeder stroom in Nederland in verband met pesticidenbesmettingen van melk. In 1977 werd een tweejarige, planteziektenkundige bedrijfsopleiding bij de Plantenziektenkundige Dienst (PD) te Wageningen met goed gevolg afgesloten. Benoeming in vaste dienst bij de PD als bacteriologisch diagnostisch medewerker volgde op 1 april 1977. In 1979 en 1980 werd met goed gevolg de HBO cursus Biologie/Botanie van het NRLO gevolgd. Met een aantal vrijstellingen werd in 1984 de studie Planteziektenkunde aan de Landbouwuniversiteit te Wageningen aangevangen. In 1986 werd het doctoraalexamen afgelegd met als hoofdvak Fytopathologie (bacteriologie) en bijvakken Microbiologie en Virologie. In 1986 volgde tevens benoeming als hoofd van de afdeling Bacteriologie van de PD. Het huidige werkveld betreft diagnose van bacteriële planteziekten en onderzoek op het gebied van biologie, detectie, classificatie en identificatie van plantepathogene bacteriën. De auteur participeert ook in onderzoek en werkgroepen in EG- en EPPO- (European Plant Protection Organisation) verband en is actief in het kader van ontwikkelingssamenwerking. Hij is lid van het bestuur van een christelijke lagere school en vertegenwoordigt deze school in het bestuur van de Schoolbegeleidingsdienst te Ede.