A NUMERICAL APPROACH TO THE TAXONOMY OF *XANTHOMONAS* AND SOME PHYTOPATHOGENIC *PSEUDOMONAS* SPP. AS DETERMINED BY PHENOTYPIC PROPERTIES AND PROTEIN GEL ELECTROPHORESIS

by

ELRITHA VAN ZYL

Submitted in partial fulfilment of the requirements
for the degree

Ph.D. (Microbiology)

in the Faculty of Science,

Department of Microbiology and Plant Pathology,

University of Pretoria, Pretoria,

Republic of South Africa.

November 1990

I declare that this thesis hereby submitted to the University of
Pretoria in fulfilment of the requirements for the degree Ph.D.
(Microbiology) has not been submitted for a degree to any other
university.

NOMINA SI NESCIS, PERIT ET COGNITIO RERUM - LINNAEUS

(If the names are neglected, the knowledge of things will
 also perish)

To my parents and Hermie

ACKNOWLEDGEMENTS

Hereby I wish to express my sincere thanks and appreciation to:
- The Tobacco Board of S.A. for granting me this opportunity through their indispensable financial support. In particular Mr. Attie Erasmus who exerted himself for this cause, and for having my interests at heart. May they also benefit from this study.

- Prof. P.L. Steyn who's idea this originally was, and to whom I am greatly indebted. For going out of his way to simplify my task in every respect, and for his enthusiasm, support, trust, motivation and patience, I will always be grateful. Especially for always being available to listen and to help, I have great appreciation.

- Johan Maritz for his personal input, the trouble taken, and his endless patience with the computer programs.

- Nico Mienie and Helga Dagutat for their invaluable support, especially in looking after my interests at the university in my absence.

- Mrs. A. van Zyl for her willingness to type this thesis.

- My parents for their valued inspiration and support over many years.

- My husband for his loyal support, patience and encouragement.

- Family, friends and colleagues for their interest, encouragement and understanding.

- The numerous people not mentioned here, who in some way contributed to this study.

---oOo---
SOLI DEO GLORIA

Note: For practical reasons, the text is presented in the format
of <u>Systematic and Applied Microbiology</u>.

CONTENTS

SUMMARY

A NUMERICAL APPROACH TO THE TAXONOMY OF <u>XANTHOMONAS</u> AND SOME
PHYTOPATHOGENIC <u>PSEUDOMONAS</u> SPP. AS DETERMINED BY PHENOTYPIC
PROPERTIES AND PROTEIN GEL ELECTROPHORESIS

by

ELRITHA VAN ZYL

PROMOTER : Prof. P.L. Steyn
DEPARTMENT: Microbiology and Plant Pathology
DEGREE FOR WHICH THESIS IS SUBMITTED: Ph.D. (Microbiology)

November 1990

    The applicability of polyacrylamide gel electrophoresis
(PAGE) of whole cell protein extracts to the classification and
rapid identification of phytopathogenic <u>Pseudomonas</u> and
<u>Xanthomonas</u> species and pathovars was investigated. Results of
numerical analysis of protein electrophoregrams were compared
with those obtained from numerical analysis of classical
phenotypic features. Shortcomings of and objections to certain
taxonomic criteria for these genera are discussed. The PAGE
technique was also tested in actual practice.

2

It is concluded that electrophoresis of the total soluble proteins of the bacterial cell is a powerful, rapid and relatively simple method, applicable to everyday use, for the differentiation and identification of phytopathogenic pseudomonads and xanthomonads up to an infrasubspecific level. Results correlate to a high degree with the clustering obtained from classical phenotypic features, as well as DNA homology and, to a certain extent, rRNA homology groupings.

Subgeneric relationships were determined by numerical taxonomy, as well as protein electrophoresis, within rRNA homology groups I, II, III and V, resulting in several proposals concerning the taxonomic positions of members of these genera.

The reliability of PAGE as a taxonomic marker to supplement and evaluate phylogenetic relationships, together with its universal applicability to phytobacteriology, is illustrated, emphasising its potential role in the proposed polyphasic approach to Proteobacteria taxonomy.

As a result of this study, the present emphasis on phylogenetic taxonomy and natural relationships between bacterial groups is questioned in view of the implications of this approach for practical, everyday phytobacteriology. The applicability of PAGE to applied phytobacteriology is illustrated by the rapid identification of a field isolate, while other advantages of PAGE for practical microbiology are also emphasised by this study.

The value and power of computer-assisted numerical analysis is demonstrated.

SAMEVATTING

'N NUMERIESE BENADERING TOT DIE TAKSONOMIE VAN XANTHOMONAS
EN SOMMIGE PLANTPATOGENE PSEUDOMONAS SPP. SOOS BEPAAL MET BEHULP
VAN FENOTIPIESE EIENSKAPPE EN PROTEïEN-JELELEKTROFORESE

deur

ELRITHA VAN ZYL

STUDIELEIER: Prof. P.L. Steyn
DEPARTEMENT: Mikrobiologie en Plantpatologie
GRAAD WAARVOOR VERHANDELING VOORGELê WORD: Ph.D. (Mikrobiologie)

November 1990

'n Ondersoek is geloods na die toepaslikheid van
poli-akrielamied-jelelektroforese van sellulêre proteïen-
ekstrakte vir die klassifikasie en vinnige identifikasie van
plantpatogene Pseudomonas en Xanthomonas spesies en patovars.
Die resultate van die numeriese analise van
proteïenelektroforegramme is vergelyk met dié van numeriese
analise van klassieke fenotipiese eienskappe. Tekortkominge van
en besware teen sekere taksonomiese maatstawwe van toepassing op
bogenoemde genera word bespreek. Hierdie elektroforetiese
tegniek is ook in die praktyk uitgetoets.

4

Daar word tot die gevolgtrekking gekom dat elektroforese van die totale oplosbare proteïeninhoud van die bakteriese sel 'n kragtige, vinnige en relatief eenvoudige metode is vir die onderskeiding en identifikasie van plantpatogene pseudomonade en xanthomonade tot op 'n infrasubspesifieke vlak. Resultate stem grootliks ooreen met groeperings verkry deur numeriese analise van klassieke fenotipiese eienskappe, asook DNA homologie, en tot 'n sekere mate, rRNA homologie groeperings.

Met behulp van numeriese taksonomie en proteïenelektroforese, is die subgeneriese verwantskappe binne rRNA- homologiegroepe I, II, III en V bepaal, wat aanleiding gee tot verskeie voorstelle rakende die taksonomiese posisies van lede van hierdie genera.

Die geloofwaardigheid van die proteïenelektroforese tegniek as 'n taksonomiese hulpmiddel om filogenetiese verwantskappe aan te vul en te evalueer, sowel as die wye toepassing moontlik in plant-bakteriologie, word geïllustreer, en beklemtoon die moontlike rol van die proteïenelektroforetiese tegniek in die voorgestelde polifasiese benadering tot die taksonomie van die klas Proteobacteria.

As uitvloeisel van hierdie studie, word die huidige beklemtoning van filogenetiese taksonomie en die natuurlike verwantskappe tussen bakteriese groepe bevraagteken in die lig van die implikasies wat hierdie benadering inhou vir praktiese, alledaagse plantbakteriologie. Die nut van proteïenjelelektroforese vir toegepaste plantbakteriologie word geïllustreer deur die vinnige identifikasie van 'n veldisolaat, terwyl ander voordele van proteïenjelelektroforese vir praktiese mikrobiologie ook deur hierdie studie beklemtoon word.

Die waarde en trefkrag van rekenaargesteunde numeriese analise word gedemonstreer.

CHAPTER I

INTRODUCTION

.

RATIONALE AND OBJECTIVES

The taxonomy of the phytopathogenic Pseudomonas and Xanthomonas species, as members of the Gram-negative bacteria, is in a transitory state and at the threshold of major development and change (DE VOS and DE LEY, 1983; DE VOS et al., 1985; WAYNE et al., 1987; STACKEBRANDT et al., 1988; MURRAY et al., 1990; VAUTERIN et al., 1990).

The development of and advances in techniques and instrumentation available to answer questions at the macromolecular level, have opened extraordinary opportunities for microbial systematics (MURRAY et al., 1990). Especially the advent of techniques for the measurement of evolutionary divergence in the structure of semantides, and the consequent development of phylogenetic taxonomy, have led to numerous changes, also in the taxonomy of the phytopathogenic bacteria (DE VOS et al., 1985; JOHNSON and PALLERONI, 1989; VAUTERIN et al., 1990).

A recent development is the introduction of the class Proteobacteria in the division Gracilicutes within the eubacteria (STACKEBRANDT et al., 1988). Encompassing a large proportion of Gram-negative bacteria, the Proteobacteria is the largest, and only formally designated, major grouping based on phylogenetic principles within the eubacteria (MURRAY et al., 1990). Pseudomonas and Xanthomonas species, constituting more than three-quarters of all phytopathogenic bacteria (STOLP et al., 1965), are included in the gamma subclass of the Proteobacteria (MURRAY et al., 1990).

Most of the taxonomic analysis performed on Pseudomonas species in recent years have, therefore, moreover emphasised the generic heterogeneity of the genus and its phylogenetic relationships with other genera (PALLERONI et al., 1973; DE VOS and DE LEY, 1983; DE VOS et al., 1985; DE VOS et al., 1989), and very few studies were carried out at the species level

to revise the phenotypic definitions of these organisms and to determine their generic positions and subgeneric relationships (GAVINI et al., 1989).

A cautionary note about hierarchical interpretation is, however, expressed by several authorities (STANIER et al., 1977; WAYNE et al., 1987; MURRAY et al., 1990). According to STANIER et al. (1977), reflection and experience have shown that the goal of a phylogenetic system of classification can seldom be realized, as the course that evolution has actually followed can be ascertained only from direct historical evidence which is contained in the fossil record, of which a complete record for the microorganisms does not exist (STALEY and KRIEG, 1984). Differences in evolutionary rates in various groups of bacteria, is presently also restricting the use of phylogenetic parameters alone in delineating taxa (STANIER et al., 1977; MURRAY et al., 1990), and should active searches for additional powerful semantides independent of the ribosomal ribonucleic acid (rRNA) cistrons be encouraged (WAYNE et al., 1987).

It is furthermore completely impracticable to define genera solely on the basis of phylogenetic data. Genera need to be characterised by using phenotypic properties, even if the choice of phenotypic markers might change given the development of better tests (MURRAY et al., 1990). A polyphasic approach (i.e. the integrated use of phylogenetic and phenotypic characteristics) is therefore advocated by several authorities (WAYNE et al., 1987; MURRAY et al., 1990; VAUTERIN et al., 1990). For this purpose, reliable taxonomic markers have to be sought e.g. polyacrylamide gel electrophoresis of cell proteins, to name but one (MURRAY et al., 1990). Greater emphasis should also be given in research to discover and recognise simple phenotypic markers that can be used to supplement and evaluate phylogenetic relationships (MURRAY et al., 1990). Again, the use of chemotaxonomic markers can be expected to help in delimiting groups of related species (MURRAY et al., 1990).

Numerical taxonomy met the need for an objective method of taxonomic analysis enabling the quantification of the similarities and differences among organisms, and is aimed at sorting individual strains of bacteria into homogeneous groups at different levels (SNEATH, 1986). The outstanding properties of numerical taxonomy are repeatability and objectivity (SOKAL and SNEATH, 1963). Numerical taxonomy is concerned primarily with phenetic relationships, but it has in recent years been extended to phylogenetic work, so as to give, from phenetic data, the most probable phylogenetic reconstructions (SNEATH, 1986).

The identification of phytopathogenic bacteria is a time-consuming process, often requiring one to several weeks of isolation and testing to make a positive identification (McINTYRE and SANDS, 1977). In at least three general situations identification of phytopathogenic bacteria is either very difficult or wellnigh impossible: when the bacteria have been isolated from a habitat having no discernible relationship to a known plant disease; when a previously unreported plant disease or symptom is involved; and when a bacterial pathogen that is unrelated to one of the common genera of phytopathogenic bacteria is the cause of a plant disease (STARR, 1981).

As the genus Xanthomonas is currently defined, yellow colour and pathogenicity are the most reliable characteristics that can be used for their presumptive identification (IREY and STALL, 1981; STARR, 1981). Although pathogenicity is of important practical value, it is of limited taxonomic use, and several authors are of the opinion that there is no reason to assign crucial importance to a single phenotypic feature such as phytopathogenicity (SCROTH and HILDEBRAND, 1983; VAUTERIN et al., 1990). Host specificity is also not regarded as a representative marker for phylogenetic classification (VAUTERIN et al., 1990), while the current pathovar system adopted for the majority of bacterial phytopathogens to indicate host specificity is regarded as unsatisfactory and that it should be replaced (SCROTH and HILDEBRAND, 1983; VAUTERIN et al., 1990). Rather,

the ultimate goal is that nomenclature shall reflect genomic phylogenetic relationships to the greatest possible extent (WAYNE et al., 1987; MURRAY et al., 1990).

For the above reasons, deoxyribonucleic acid (DNA) homology was established as the standard arbiter for the designation of species, and was a molecular definition of species recommended (MURRAY et al., 1990). The advantage of adopting this or a similar restrictive species definition must be weighed against its potential impact on well established and accepted bacterial groups. For practical reasons, classifications and nomenclature should remain stable (although not static), because changes create confusion, particularly at the genus and species levels, and result in costly modifications of identification schemes and tests (STALEY and KRIEG, 1984). In this regard, WOESE et al. (1984) are of the opinion that the DNA-rRNA approach should soon become obsolete, because it offers neither the precision nor the extent of data obtainable with the partial sequencing or full sequencing approaches, as it samples only a small portion of the genome (0,3 - 0,5%) (KANDLER and SCHLEIFER, 1980). On the other hand, PALLERONI (1986) is of the opinion that the idea of constructing phylogenetic schemes based exclusively on sequence data has not gained universal approval, and that evidence suggests that bacterial evolutionary trees built on protein or rRNA sequence data may be invalid.

Above all, such parameters as DNA homology, DNA-rRNA homology and rRNA sequencing are difficult to apply in the classification and routine identification of bacteria (GARRETT, 1982; OYAIZU and KOMAGATA, 1983). Successfully introduced diagnostic tests are those that are relatively easy to perform, and are rapid and sensitive (McINTYRE and SANDS, 1977; GARRETT, 1982). A classification that is of little use to microbiologists, no matter how fine a scheme or who devised it, will soon be ignored or significantly modified (STALEY and KRIEG, 1984).

It is, however, also important that phytobacteriology keeps up with the modern trend and developments in bacterial systematics. Although it is so essential, recognition of the importance of effective taxonomic understanding to all fields, basic and applied, has been slow to develop (WAYNE et al., 1987). It seems that the potential of molecular methods, such as computer-assisted protein analysis, to analyse subgeneric hetergeneity among phytopathogenic bacteria that might have taxonomic significance, has not been fully developed (CIVEROLO, 1981; STARR, 1981). MURRAY et al. (1990) and VAUTERIN et al. (1990) have also foreseen a substantial role for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins (amongst others) in the proposed systematic, poly-phasic and comprehensive approach to Proteobacteria taxonomy.

The aims of this study were: (i) To investigate the applicability of polyacrylamide gel electrophoresis of cellular proteins for the differentiation and rapid identification of phytopathogenic Pseudomonas and Xanthomonas species and pathovars, with and without computer assistance; (ii) To compare the results of numerical analysis of protein electrophoregrams with those obtained from classical numerical taxonomy, as well as other molecular methods; (iii) To determine the subgeneric relationships of representative strains of phytopathogenic Pseudomonas and Xanthomonas species of four rRNA homology groups in view of the proposed polyphasic approach; (iv) To evaluate several criteria presently used for classification and identification in the genera Pseudomonas and Xanthomonas; (v) To determine the applicability of PAGE to applied phytobacteriology.

This dissertation is a document of the approaches followed to pursue the aims set out above, of the results obtained and the conclusions arrived at.

REFERENCES

CIVEROLO, E.L.: New developments in microbial identification and taxonomy, pp. 83-84. In: Proceedings of the fifth international conference on plant pathogenic bacteria (J.C. LOZANO, ed.). Cali, CIAT 1981

DE VOS, P., DE LEY, J.: Intra- and intergeneric similarities of Pseudomonas and Xanthomonas ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 33, 487-509 (1983)

DE VOS, P., GOOR, M., GILLIS, M., DE LEY, J.: Ribosomal ribonucleic acid cistron similarities of phytopathogenic Pseudomonas species. Int. J. system. Bact. 35, 169-184 (1985)

DE VOS, P., VAN LANDSCHOOT, A., SEGERS, P., TYTGAT, R., GILLIS, M., BAUWENS, M., ROSSAU, R., GOOR, M., POT, B., KERSTERS, K., LIZZARAGA, P., DE LEY, J.: Genotypic relationships and taxonomic localization of unclassified Pseudomonas and Pseudomonas-like strains by deoxyribonucleic acid: ribosomal ribonucleic acid hybridizations. Int. J. system. Bact. 39, 35-49 (1989)

GARRETT, C.M.E.: Bacterial diseases of food plants - an overview, pp. 115-132. In: Bacteria and plants (M.E. RHODES-ROBERTS, F.A. SKINNER, eds.). London, Academic Press 1982.

GAVINI, F., HOLMES, B., IZARD, D., BEJI, A., BERNIGAUD, A., JAKUBCZAK, E.: Numerical taxonomy of Pseudomonas alcaligenes, P. pseudoalcaligenes, P. mendocina, P. stutzeri, and related bacteria. Int. J. system. Bact. 39, 135-144 (1989)

IREY, M.S., STALL, R.E.: Value of xanthomonadins for identification of pigmented Xanthomonas campestris pathovars, pp. 85-95. In: Proceedings of the fifth international conference on plant pathogenic bacteria (J.C. LOZANO, ed.). Cali, CIAT 1981

JOHNSON, J.L., PALLERONI, N.J.: Deoxyribonucleic acid similarities among Pseudomonas species. Int. J. system. Bact. 39, 230-235 (1989)

KANDLER, O., SCHLEIFER, K-H: Systematics of bacteria, pp. 234-247. In: Progress in botany, Fortschritte der botanik (H. ELLENBERG, K. ESSER, K. KUBITZKI, E. SCHNEPF, H. ZIEGLER, eds.), vol. 42. Berlin, Springer-Verlag 1980

McINTYRE, J.L., SANDS, D.C.: How disease is diagnosed, pp. 35-53. In: Plant disease, an advanced treatise (J.G. HORSFALL, E.B. COWLING, eds.), vol. 1. London, Academic Press 1977

MURRAY, R.G.E., BRENNER, D.J., COLWELL, R.R., DE VOS, P.,
GOODFELLOW, M., GRIMONT, P.A.D., PFENNIG, N., STACKEBRANDT, E.,
ZAVARZIN, G.A.: Report of the ad hoc committee on approaches
to taxonomy within the proteobacteria. Int. J. system. Bact.
40, 213-215 (1990)

OYAIZU, H., KOMAGATA, K.: Grouping of Pseudomonas species on the
basis of cellular fatty acid compositions and the quinone
system with special reference to the existence of 3-hydroxy
fatty acids. J. gen. appl. Microbiol. 29, 17-40 (1983)

PALLERONI, N.J.: Taxonomy of the pseudomonads, pp. 3-25. In:
The bacteria: the biology of Pseudomonas (J.R. SOKATCH, ed.).
London, Academic Press 1986

PALLERONI, N.J., KUNISAWA, R., CONTOPOULOU, R., DOUDOROFF, M.:
Nucleic acid homologies in the genus Pseudomonas. Int. J.
system. Bact. 23, 333-339 (1973)

SCROTH, M.N., HILDEBRAND, D.C.: Toward a sensible taxonomy of
bacterial plant pathogens. Plant Disease 67, 128 (1983)

SNEATH, P.H.A.: Numerical taxonomy, pp. 969-971. In: Bergey's
manual of systematic bacteriology (P.H.A. SNEATH, N.S. MAIR,
M.E. SHARPE, J.G. HOLT, eds.), 9th ed., vol. 2. London,
Williams and Wilkins 1986

SOKAL, R.R., SNEATH, P.H.A.: Principles of numerical taxonomy. San Francisco, W.H. Freeman and Co. 1963

STACKEBRANDT, E., MURRAY, R.G.E., TRÜPER, H.G.: _Proteobacteria_ classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives". Int. J. system. Bact. <u>38</u>, 321-325 (1988)

STALEY, J.T., KRIEG, N.R.: Classification of procaryotic organisms: an overview, pp. 1-3. In: Bergey's manual of systematic bacteriology (N.R. KRIEG, J.G. HOLT, eds.), 9th ed., vol. 1. Baltimore, Williams and Wilkins 1984.

STANIER, R.Y., ADELBERG, E.A., INGRAHAM, J.L.: General microbiology. London, The Macmillan Press 1977

STOLP, H., STARR, M.P., BAIGENT, N.L.: Problems in speciation of phytopathogenic pseudomonads and xanthomonads. Ann. Rev. Phytopath. <u>3</u>, 231-264 (1965)

STARR, M.P.: The genus _Xanthomonas_, pp. 742-763. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (M.P. STARR, H. STOLP, H.G. TRÜPER, A. BALOWS, H.G. SCHLEGEL, eds.). Berlin, Springer-Verlag 1981

VAUTERIN, L., SWINGS, J., KERSTERS, K., GILLIS, M., MEW, T.W., SCHROTH, M.N., PALLERONI, N.J., HILDEBRAND, D.C., STEAD, D.E., CIVEROLO, E.L., HAYWARD, A.C., MARAîTE, H., STALL, R.E., VIDAVER, A.K., BRADBURY, J.F.: Towards an improved taxonomy of Xanthomonas. Int. J. system. Bact. 40, 312-316 (1990)

WAYNE, L.G., BRENNER, D.J., COLWELL, R.R., GRIMONT, P.A.D., KANDLER, O., KRICHEVSKY, M.I., MOORE, L.H., MOORE, W.E.C., MURRAY, R.G.E., STACKEBRANDT, E., STARR, M.P., TRÜPER, H.G.: Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. system. Bact. 37, 463-464 (1987)

WOESE, C.R., BLANZ, P., HAHN, C.M.: What isn't a pseudomonad: the importance of nomenclature in bacterial classification. System. Appl. Microbiol. 5, 179-195 (1984)

CHAPTER II

DIFFERENTIATION OF PHYTOPATHOGENIC PSEUDOMONAS AND XANTHOMONAS
SPECIES AND PATHOVARS BY NUMERICAL TAXONOMY AND PROTEIN GEL
ELECTROPHOREGRAMS

SUMMARY

The applicability of numerical analysis of polyacrylamide gel electrophoregrams of whole cell protein extracts for the classification and rapid identification of phytopathogenic Pseudomonas and Xanthomonas species and pathovars was investigated and compared with numerical analysis of phenotypic features. It is concluded that electrophoresis of the total soluble proteins of the bacterial cell is a powerful, rapid and relatively easy method, applicable to everyday phytobacterial use, for the differentiation of phytopathogenic pseudomonads and xanthomonads up to a sub-specific level. Results correlated to a high degree with the clustering obtained from classical phenotypic features. On the basis of this study several taxonomic proposals, concerning this group of organisms, are made.

INTRODUCTION

The genera Pseudomonas Migula 1894 and Xanthomonas Dowson 1939 are of substantial economic importance, as members of these genera constitute more than three-quarters of all phytopathogenic bacteria (STOLP et al., 1965). The taxonomy of these phytopathogens and their interrelationships are not yet fully resolved, often presenting practical difficulties to those involved in the routine classification and identification of strains of these genera (COLWELL and LISTON, 1961; LYSENKO, 1961; DE LEY et al., 1965; STOLP et al., 1965; SANDS et al., 1970; PALLERONI et al., 1973; PALLERONI, 1981; SCHROTH et al., 1981; STARR, 1981; DE VOS et al., 1983; OYAIZU et al., 1983; PALLERONI, 1984; DE VOS et al., 1985).

The main body of the present classification of Pseudomonas species is based upon the existence of five rRNA homology groups of named pseudomonads (PALLERONI et al., 1973; DE VOS et al., 1985). These rRNA groups are less related to each other than they are to other Gram-negative genera (DE VOS et al., 1985).

One of these groups (rRNA group V) contains members of the genus
Xanthomonas.

    The present generic definitions of Xanthomonas and Pseudomonas
do not allow a sharp separation between these two genera (DE VOS
et al., 1985). Plant pathogenicity and the production of
xanthomonadins are two important distinctive properties of the
xanthomonads, but otherwise these organisms are typical
pseudomonads (PALLERONI, 1984), and therefore unequivocal
identification in either one of these genera is difficult for
some of these strains (DE VOS et al., 1985). Various sorts of
relationships exist between pseudomonads and xanthomonads (STARR,
1981), and some members of these two genera actually share a
substantial degree of DNA-rRNA homology (PALLERONI, 1984).
PALLERONI (1984) concluded that, from a practical determinative
standpoint, a clear-cut circumscription of the family
Pseudomonadaceae by classical phenotypic criteria has become more
difficult than ever before, and that differentiation of the
genera may require specialised techniques which are still beyond
the reach of many laboratories involved in problems of
determinative bacteriology.

    Phytopathogenic Pseudomonas species are allocated in three of
the five rRNA homology groups, namely groups I, II and III (DE
VOS et al., 1985; WILLEMS et al., 1987). Solid bacteriological
criteria for the differentiation of species and pathovars from
one another and from saprophytic forms are still needed and,
consequently, strains isolated from sources other than plant
lesions seldom can be assigned with certainty to described
species or pathovars (PALLERONI, 1984). Few phenotypic
characters are taxonomically useful (PALLERONI, 1984).

    The taxonomic situation in the genus Xanthomonas at the
generic, specific, and sub-specific levels has been explored by a
variety of approaches, but still the problem of subdividing the
xanthomonads has no satisfactory solution at the present moment
(PALLERONI, 1981). The opinion of BURKHOLDER and STARR (1948),

DYE (1962) and FRIEDMAN and DE LEY (1965) that it may be wellnigh impossible to identify Xanthomonas species by laboratory methods, has thus remained fundamentally unchanged.

The overriding taxonomic consideration behind assignment of bacteria to the genus Xanthomonas is an ecological feature, namely to cause plant disease (STARR, 1981). However, this practice of forming a species solely on the basis of the host of original isolation has commonly been found to be unsatisfactory. It fails to allow the identification of avirulent strains or the easy recognition of organisms isolated from various sources or of unknown origin (DYE, 1962). For this reason, possible taxonomic relationships among Xanthomonas pathovars are still elusive. More than 125 different pathovars of X. campestris are currently recognized and the primary means of differentiating them is by means of the plant host(s) of that pathovar, although the potential host ranges of most pathovars are largely unknown (LAZO and GABRIEL, 1987). Further, and despite a widely held view that all xanthomonads are plant pathogens (SCHROTH and HILDEBRAND, 1983), nonpathogenic members of X. campestris are sometimes found as ectoparasitic leaf colonizers (ERCOLANI, 1978) and occasionally as endoparasites (MULREAN et al., 1982; MAAS et al., 1985). Their inability to provoke a pathogenic response renders them unclassifiable in this system (LAZO et al., 1987). The next level of decision regarding membership in the genus Xanthomonas generally stems from the yellow colour of the colonies when grown on sugar-containing media (STARR, 1981). Colourless xanthomonads, however, do exist and pose the usual systematic dilemma which emerges when too great reliance is placed upon a single determinative trait (STARR and STEPHENS, 1964; HAYWARD, 1966; DE VOS et al., 1985). It would be helpful if alternative means to differentiate among X. campestris pathovars were available (LAZO and GABRIEL, 1987).

The widespread exchange of plant material and its fast intercontinental transport make imperative the use of rapid and reliable tests for identification of plant pathogenic bacteria.

Accurate identification of a pathogen is also a necessary prerequisite to control (GARRETT, 1982). Parameters such as DNA-DNA homology (PALLERONI et al., 1972), DNA-rRNA homology (PALLERONI et al., 1973; DE VOS et al., 1985), oligonucleotide cataloging of 16S ribosomal RNA (WOESE et al., 1984) and restriction fragment-length polymorphism (RFLP) (LAZO et al., 1987), which have all been used in taxonomic studies of the pseudomonads and xanthomonads, are often difficult to apply in the routine classification and identification of bacteria (GARRETT, 1982; OYAIZU et al., 1983). Of P. solanacearum it is said that the wide variation in the characteristics of 'isolates of this pathogen has discouraged research workers from the all-important identification of strains which is so necessary for the determination of varietal resistance and disease control (GARRETT, 1982). More rapid and reliable tests for identifying pathogens will speed their recognition and help pathologists and growers to take appropriate action more quickly (GARRETT, 1982).

Evaluation of the available methods for the routine classification and identification of the phytopathogenic pseudomonads and xanthomonads revealed the following: the API 20NE commercial biochemical identification kit is presently unsatisfactory as it cannot yet differentiate between Pseudomonas pathovars nor Xanthomonas species or pathovars (API analytical profile index, 1984). DNA base composition (G + C contents) is of limited use because of the overlapping values for Pseudomonas (58-70 mol% G + C) and Xanthomonas (63-71 mol% G + C) (DE LEY et al., 1966; PALLERONI et al., 1973). STOLP et al. (1965) also reported that members of a genus cannot be subdivided into species on the basis of G + C values. DE VOS et al. (1985) reported the same for DNA-rRNA hybridizations, namely that this method generally does not allow species separation within a rRNA branch. The use of monoclonal antibodies showed considerable promise (ALVAREZ et al., 1985) although FOX (1987) is of the opinion that detection methods such as those based on DNA probes or monoclonal antibodies tend to focus on single traits and thus may often give negative results which are difficult to interpret.

Serology (GARRETT, 1982; PALLERONI, 1984) and phage typing (BILLING and GARRETT, 1980; PALLERONI, 1984) were not very successful as taxonomic tools in Xanthomonas. Although differential fatty acid patterns produce results relatively fast, the technique is often unpopular because of the expensive, and frequently temperamental, gas-chromatographic detection devices required (FOX, 1987). STARR's (1981) opinion that workable criteria are badly needed to unambiguously distinguish the genus Pseudomonas from the genus Xanthomonas (and species and pathovars within these genera), is therefore still relevant.

One technique of classification and identification at a molecular level that has shown considerable promise, is the chemotaxonomic technique of polyacrylamide gel electrophoresis of the total soluble proteins of the bacterial cell. EL-SHARKAWY and HUISINGH (1971) found the gel electrophoretic technique a very useful tool for the taxonomic differentiation of phytopathogenic xanthomonads, that were at that time separable only on the basis of their specific pathogenicity. STARR (1981) was surprised that this significant advance, which moreover involves relatively simple procedures, had not been followed up, especially because of its likely bearing on phytopathogenic specialisation. PAGE also proved useful in differentiating among X. campestris pathovars (VERA CRUZ et al., 1984; VAN DEN MOOTER et al., 1987). PALMER and CAMERON (1971) electrophoretically compared several phytopathogenic pseudomonads and also concluded that electrophoresis should be of value as a relatively rapid and simple method of identification of Pseudomonas species.

The major expression of the microbial genome results in the synthesis of about 2000 different protein molecules in the microbial cell. These molecules form an information source of immense potential and richness for the characterisation of micro-organisms (JACKMAN, 1985). Zone electrophoresis of these proteins under well-defined standardised conditions produces protein banding patterns (electrophoregrams) that can be considered as "fingerprints" of the bacterial strains under

investigation (KERSTERS and DE LEY, 1980). Electrophoretic methods of suitable resolution and reproducibility thus provide a basis for microbial systematics that is objective, rapid and relevant to many laboratories (JACKMAN, 1985).

In recent years this method, which is moreover relatively simple and inexpensive, has been extensively used for the classification and identification of bacteria (PALMER and CAMERON, 1971; KERSTERS and DE LEY, 1980). When used to screen isolates, this procedure reduced total analytical time and expense without sacrificing accuracy (MOORE et al., 1980). In many instances, the pattern obtained within 24 h of isolating an organism was sufficiently distinctive so that the identity of the organism could be strongly suspected (KRIEG and JONES, 1986). While the results of this method correlate well with those of DNA-DNA hybridizations (KERSTERS and DE LEY, 1980; JACKMAN, 1982; OWEN and JACKMAN, 1982) and numerical analysis of phenotypic features (KERSTERS and DE LEY, 1975; VERA CRUZ et al., 1984), it is usually considerably faster.

As the ideal test should identify a specific pathogen rapidly and unambiguously, the above indicated that electrophoresis of the total soluble proteins of bacterial cells would be the method of choice for the differentiation and rapid identification of phytopathogenic Pseudomonas and Xanthomonas species and pathovars in everyday phytobacterial practice.

The aims of this study were therefore:
(1) To investigate the applicability of numerical analysis of polyacrylamide gel electrophoregrams for the differentiation and identification of phytopathogenic Pseudomonas and Xanthomonas species and pathovars.
(2) To compare the results as obtained with electrophoresis with those obtained from numerical analysis of classical phenotypic features of the same group of organisms.

MATERIALS AND METHODS

Bacterial strains

A list of the bacterial strains used in this study appears in Table 1. Twenty of the Pseudomonas and 10 of the Xanthomonas strains were from the National Collection of Plant Pathogenic Bacteria (Harpenden, England). Avirulent strains of X. campestris pv. mangiferaeindicae, of which two (0823 and 0836) were used in this study, were received from Dr. M.L. Moffet (Australia). The strains of P. solanacearum from tobacco and tomatoes were kindly supplied by M.C. Engelbrecht (TCRI, S.A.) and those from potatoes by A. Swanepoel (VOPRI, S.A.). UP 89 was isolated from bacterial black spot of mangoes by N. Viljoen (1972) and identified as P. mangiferaeindicae (STEYN et al., 1974). One of the X. campestris pv. mangiferaeindicae strains (D42) was a local isolation from gall lesions on mango leaves (VAN ZYL et al., 1988).

Culture conditions

Strains were grown at 28°C on GYCA medium (VERA CRUZ et al., 1984). With the exception of P. solanacearum strains, that were maintained in sterile water at room temperature (15-25°C), all strains were maintained on GYCA slants, also at room temperature. Strains were transferred to fresh medium every 2 to 3 weeks and checked for purity by plating and by examination of living and Gram-stained cells. All isolates were also freeze-dried.

For electrophoretic studies, cultures were shaker-incubated at 28°C and grown to saturation in 200 ml Std 1 nutrient broth (Merck) supplemented with 0,15% K_2HPO_4 and 0,15% $MgSO_4.7H_2O$.

Morphological, physiological and biochemical features

The following tests were performed according to DYE (1962): Gram-staining of 24 to 48 h GYCA cultures; motility; colour and

Legend to Table 1. Strains used for phenotypic tests and protein gel electrophoresis.

a) ATCC, American Type Culture Collection, Rockville, Maryland;
   NCPPB, National Collection of Plant Pathogenic Bacteria,
   Harpenden, England;
   PDDCC, Culture Collection of the Plant Disease Division,
   Department of Scientific and Industrial Research, Auckland,
   New Zealand;
   TCRI, Tobacco and Cotton Research Institute, Kroondal,
   South Africa;
   VOPRI, Vegetable and Ornamental Plant Research Institute,
   Pretoria, South Africa.

b) Omitted from the Approved Lists of Bacterial Names (SKERMAN
   et al., 1980).

c) P. marginalis pv. marginalis; not included in electrophoretic
   studies.

d) P. marginalis pv. alfalfae.

e) Two colony types were found for these strains. Only the
   irregularly-round, white, fluidal colonies were used for
   phenotypic tests, while both the white, mucoid type (t1)
   and the butyrous, brown type (t2) were used for
   electrophoresis.

f) Supplied by M.C. Engelbrecht, TCRI, S.A.

g) Supplied by A. Swanepoel, VOPRI, S.A.

h) Supplied by Dr.M.L. Moffett, Queensland, Australia.

i) Isolated in 1972 by N. Viljoen.

j) Formerly Xanthomonas ampelina. Reclassified as Xylophilus
   ampelinus by WILLEMS et al. (1987).

Table 1. Strains used for phenotypic tests and protein gel electrophoresis.

PHENON	STRAIN NO[a]	SPECIES/PATHOVAR NAME	OTHER STRAIN DESIGNATIONS	HOST PLANT	LOCALITY OF ORIGIN (IF NOT FROM CULTURE COLLECTION)
I	NCPPB 2653	P. aeruginosa	ATCC 10145		
	ATCC 13453	P. angulata[b]		Nicotiana tabacum	
	NCPPB 1011	P. avenae	ATCC 19860	Avena sativa	
	NCPPB 1873	P. caricapapavae		Carica papava	
	NCPPB 1964	P. fluorescens bv. I	ATCC 13525		
	NCPPB 667	P. fluorescens bv. II[c]	ATCC 10844	Lactuca sativa	
	NCPPB 2644	P. fluorescens bv. II[d]	PDDCC 5708	Medicago sativa	
	NCPPB 1797	P. fluorescens bv. III			
	NCPPB 325	P. solanacearum	ATCC 11696	Lycopersicon esculentum	
	TCRI 01	P. solanacearum[e]		Nicotiana tabacum	Kroondal, S.A.[f]
	TCRI 02	P. solanacearum[e]		Lycopersicon esculentum	Kroondal, S.A.[f]
	VOPRI 5	P. solanacearum[e]		Solanum tuberosum	Clanwilliam, S.A.[g]
	VOPRI 26	P. solanacearum[e]		Solanum tuberosum	Makoppa, S.A.[g]
	NCPPB 2356	P. syringae pv. mellea	PDDCC 5711	Nicotiana tabacum	
	NCPPB 2995	P. syringae pv. morsprunorum	ATCC 19322	Prunus spp.	
	NCPPB 639	P. syringae pv. savastanoi	ATCC 13522	Olea europaea; Fraxinus spp.	
	NCPPB 281	P. syringae pv. syringae	ATCC 19310	Syringa vulgaris	
	NCPPB 1427	P. syringae pv. tabaci	PDDCC 2835	Nicotiana tabacum	
	NCPPB 2192	P. tolaasii		Cultivated mushrooms	
	NCPPB 635	P. viridiflava	ATCC 13223	Phaseolus vulgaris	
II	NCPPB 457	X. axonopodis	ATCC 19312	Axonopus spp.	
	NCPPB 528	X. campestris pv. campestris	PDDCC 13	Brassica spp.	
	NCPPB 101	X. campestris pv. cassavae	PDDCC 204	Manihot spp.	
	NCPPB 490	X. campestris pv. mangiferaeindicae	PDDCC 5740	Mangifera indica	
	0823	X. campestris pv. mangiferaeindicae		Mangifera indica	Nambour, Queensland[h]
	0836	X. campestris pv. mangiferaeindicae		Mangifera indica	Gin Gin, Queensland[h]
	UP 89	"P. mangiferaeindicae"[i]		Mangifera indica	Northern Transvaal, S.A.
	D42	X. campestris pv. mangiferaeindicae		Mangifera indica	Eastern Transvaal, S.A.
	NCPPB 1834	X. campestris pv. manihotis	PDDCC 5741	Manihot spp.	
	NCPPB 3035	X. campestris pv. phaseoli	PDDCC 5834	Phaseolus spp.	
	NCPPB 416	X. campestris pv. pruni	PDDCC 51	Prunus spp.	
	NCPPB 2475	X. campestris pv. viticola	PDDCC 3867	Vitis vinifera	
	NCPPB 1469	X. fragariae	PDDCC 5715	Fragaria vesca	
III	NCPPB 1683	P. hibiscicola	ATCC 19867		
	NCPPB 1974	X. maltophilia	ATCC 136237		
Not de-signa-ted	NCPPB 2607	P. amygdali		Prunus dulcis	
	NCPPB 1962	P. cepacia	ATCC 25416	Allium cepa	
	NCPPB 2217	Xy. ampelinus[j]	PDDCC 4298	Vitis vinifera	

slime formation (on GYCA only); urease production using double
sets of controls (with urea, not inoculated and without urea,
inoculated); utilisation of asparagine as the sole source of
carbon and nitrogen (using L-asparagine); catalase production;
gelatin hydrolysis with stab inoculations into nutrient gelatin
(Difco); indole production; acetoin production; nitrate
reduction; hydrogen sulphide production using yeast extract-
salts broth and 0,01% cysteine hydrochloride with the presence of
H_2S indicated by lead acetate strips (Whatman No. 1 paperstrips
soaked in 10% lead acetate solution); hydrolysis of starch using
yeast extract nutrient agar and 0,2% (w/v) soluble starch (Difco)
and flooding with a dilute iodine solution after 2 d; hydrolysis
of aesculin (results recorded up to 30 d after inoculation);
tyrosinase activity recorded after 4 d; for the oxidative
production of acid from carbohydrates and related carbon sources,
the following carbon sources were tested: L(+)-arabinose, D-
xylose, D-ribose, D-glucose, D-mannose, D-galactose, fructose,
sucrose, trehalose, cellobiose, maltose, lactose, raffinose,
melezitose, melibiose, rhamnose, starch, dulcitol, meso-inositol,
mannitol, adonitol, sorbitol, erythritol, dextrin, inulin,
salicin, glycerol, glycogen and α-methyl-D-glucoside. Carbon
sources were added aseptically from filter-sterilized,
concentrated solutions. Cultures were examined for growth and
acid production after 2, 4 and 7 days, then at 7-day intervals to
42 days.

KOVAC'S (1956) reagent was used for the oxidase test. The
production of a fluorescent pigment was tested on King, Ward and
Raney's medium B (HARRIGAN and McCANCE, 1966). The oxidation-
fermentation (O/F) test was performed according to HUGH and
LEIFSON (1953), using glucose as a carbon source. Hydrolysis of
Tween 80 (an oleic acid ester) was tested according to SIERRA
(1957). β-galactosidase activity was detected by the ONPG test
(HAYWARD, 1977). Hydrolysis of arginine with the formation of
ammonia was tested on THORNLEY'S semi-solid arginine medium
(1960). Protein digestion in liquid and agar medium was
performed according to DYE (1980), using "purple milk" and milk

agar plates. Poly-β-hydroxybutyrate accumulation was observed by phase contrast microscopy of Sudan black B stained smears according to SANDS et al. (1980). Xanthomonadin production was observed visually on GYCA medium, and results were confirmed from the literature (PALLERONI, 1984).

The suspensions used for the inoculation of all media were prepared from 48 h GYCA cultures and were standardised spectrocolorometrically to 10^7 cells/ml. Liquid media were inoculated by the addition of two drops from a sterile pipette, while one loopful of the suspension was streaked on solid media. Unless otherwise stated, incubation times were as specified by the different authors. Incubation temperature was always 28°C, except for gelatinase (25°C). All liquid media were shaker incubated.

One strain each of <u>Escherichia coli</u> and <u>Bacillus cereus</u> was used as controls.

<u>Numerical analysis of phenotypic features</u>

Fifty-three phenotypic features were determined for each of the 38 strains. All phenotypic features were coded as 1 (positive) or 0 (negative). There were no missing features and dubious results were repeated. Similarity coefficients (S_{SM}) (SOKAL and MICHENER, 1958) were calculated and the strains were clustered by unweighted average linkage (SNEATH and SOKAL, 1973) using a program in Basic written by Dr. J.J. Bezuidenhout (University of Pretoria) and an IBM Personal Computer.

<u>Polyacrylamide gel electrophoresis of soluble proteins</u>:

<u>Preparation of whole cell extracts</u>

Cells were harvested from fully grown cultures by centrifugation, washed in sterile, distilled water and centrifuged again. Cultures were checked for purity at harvest

by microscopy and streaking on GYCA plates. Harvested cells were stored at -12°C in sterile screw-cap bottles (vol. 20 ml).

When ready to commence with electrophoresis, 0,5 g (wet weight) of cells from each strain was resuspended in 15 ml sample buffer (0,0625 M Tris-HCl, pH 6,8; 2% sodium dodecyl sulphate (SDS); 10% glycerol; 5% 2-mercaptoethanol; 0,001% bromophenol blue) (LAEMMLI, 1970) and sonicated with a Dawe soniprobe (Type 7530A) with an 80 watt output for 2 min in 20 s periods, alternating with 5 to 8 s pauses for cooling. Throughout, the cell suspensions were submerged in ice to inhibit proteases. The sonicated suspensions were then heated for 2 min in a boiling waterbath to ensure denaturation of the proteins. Thereafter, 1,5 ml of the crude sonicate was centrifuged for 2 min in a bench-top centrifuge to remove the cellular debris and mucus which originates from the slimy growth of some of these strains. The final supernatant contained the total soluble proteins used for electrophoresis. Protein concentrations of the supernatants were standardised according to STEGEMANN et al. (1987). Prepared samples were used as fresh as possible. Used samples and newly prepared samples for which storation was unavoidable, were stored at -12°C.

Standard conditions for polyacrylamide gel electrophoresis

The discontinuous sodium dodecyl sulphate (SDS) buffer system of LAEMMLI (1970) was used as a basis for one dimensional slab gel electrophoresis in a Protean II vertical electrophoresis unit from Bio-Rad. Preparation, casting, assembling and running of the gels were performed according to the Protean II Slab Cell Instruction Manual (Bio-Rad, 1984). Gels were 1,5 mm thick and 160 mm long. Separating gels (10,0% acrylamide; 0,375 M Tris, pH 8,8) were prepared from a stock solution of 29,2 g acrylamide (Electran) and 0,8 g N'N'-Bis-methylene-acrylamide (Bio-Rad) made up to 100 ml with distilled water. The stacking gel (4,0% acrylamide; 0,125 M Tris, pH 6,8) was prepared from the same stock solution. The final concentration of SDS was 0,1% in both

gels. A Tris-glycine electrode buffer (0,025 M Tris, 0,192 M glycine, 0,5% SDS, pH 8,3) was used. All buffers and stock solutions were stored at 4°C but used at room temperature. Gels were cast one day prior to the electrophoretic run and left overnight at room temperature to ensure complete polymerization.

Fourteen samples (containing approximately 100 µg of soluble protein/20 µl each) (HAMES, 1981) were loaded into 14 of the 15 sample wells on one gel with a Hamilton syringe. One well was loaded with the reference proteins, namely 10 µl bovine thyroglobulin (mol wt:669000; Type I, Sigma; 0,01 g/ml) and 5 µl ovalbumin (mol wt:43000; Grade V, Sigma; 0,01 g/ml) suspended in sample buffer. 5 µl Lysozyme (mol wt:14300; 0,01 g/10 ml sample buffer) was added to each well as a bottom reference point on all the electrophoregrams. These reference proteins enabled accurate comparisons between protein patterns on different gels. A bromophenol blue solution containing 50% (v/v) glycerol was added as a tracking dye during electophoresis. Two gels were run simultaneously. Electophoresis of the proteins of all the strains was performed repeatedly until satisfactory and reproducible results were obtained.

Electrophoresis was performed in an anodic system at a constant current of 25 mA/gel for the stacking gel and 35 mA/gel for the separating gel. Water at a constant temperature of 15°C was circulated through the cooling core for the whole duration of the run. Running time was 5 to 6 hours.

Gels were fixed and stained overnight according to Anderson's Brilliant Blue R staining procedure (ANDERSON and ANDERSON, 1977) using Coomassie Brilliant Blue R-250 in ethanol and 10% acetic acid. Destaining was done in four successive steps according to the same procedure, using 5% acetic acid and 95% ethanol.

Spectrophotometry and normalization of spectrophotometric tracings

Destained gels were scanned in a Beckman DU-8 spectrophoto-
meter with a gel scan module adjusted to the following settings:
absorption, wavelength 560 nm ($E_{1cm}^{1\%}$ R-250: = 560 nm); average
reading 1,0; slit width 1,0 nm; gel slit width 0,2 mm; span
1,00; chart speed 30,0 cm/min; gel speed 10,0 cm/min; gel end
140,0 mm. The bottom centimetre of the gel was used as a blank.

Normalization of the spectrophotometric scans was performed by
the method of KERSTERS and DE LEY (1975). Working on a light
table, the spectrophotometric tracing of each sample and the
tracing of the reference proteins on the corresponding gel were
superimposed, using the top of the gels and the position of
lysozyme as points of concurrence. The positions of the
reference proteins were then marked on the sample scan. With the
aid of a computer drawn sliding scale, the distance between
thyroglobulin and ovalbumin was divided into 90 equal parts
(positions) on the x-axis, extended to 180 positions for the
whole scan. The height in mm (equivalent to the optical density
on the y-axis) of each position on the scan was measured,
converting the scan into a sequence of 180 numbers for the
computer-assisted analysis. When external factors caused slight
differences between a group of very similar protein patterns,
the best fit between each pair of traces was obtained by
laterally shifting one trace with respect to the other in single
point steps of approximately 5 points on either side of the
initial alignment with respect to the reference proteins (OWEN
and JACKMAN, 1982).

Numerical analysis of electrophoregrams

The Pearson product-moment correlation coefficient, r, (SOKAL
and SNEATH, 1963) between any pair of normalized spectrophoto-
metric tracings of protein patterns was calculated using the
heights already mentioned (KERSTERS and DE LEY, 1975). The

resulting r-matrix was transformed to a distance matrix (ROHLF and SOKAL, 1965) and clustered by the unweighted average pair-group method (UPGMA) (ZUPAN, 1982). The distance values of all clustering levels were again transformed to r-values. All calculations were performed using the IBM-mainframe computer of the Institute for Computer Sciences, University of Pretoria.

Photopgraphy and drying of gels

Gels were photographed on a light table with a white perspex plate uniformly illuminated from underneath. A Canon AE-1 camera fitted with a macro lens (28 mm focal length) and Kodak Ektachrome 50 tungsten film was used.

Gels were soaked in an aqueous solution of 1% glycerol – 10% acetic acid for 2 d and then dried between two layers of cellophane (boiled beforehand in a 5% Na_2CO_3-50 mM EDTA solution) on a Bio-Rad Gel Slab Dryer (Model 224) and filed for later reference.

RESULTS

Morphological, physiological and biochemical features

All Pseudomonas and Xanthomonas strains grew satisfactorily to abundantly on GYCA medium. The characteristic yellow-pigmented growth of most of the xanthomonads was enhanced by this medium. However, the extremely mucoid growth of the Xanthomonas strains and several Pseudomonas strains on this glucose containing medium hampered their handling and sub-culturing. P. solanacearum strains produced a diffusible brown pigment on this medium. P. solanacearum strains as well as Xy. ampelinus grew slowly and lost their viability very fast, especially when stored at 4°C.

Two colony types were found for Xy. ampelinus and four of the P. solanacearum strains. In the case of Xy. ampelinus, the two types differed in growth rate, with the slow growing colony type

Legend to Table 2. Differential phenotypic features.

(a) P. marginalis pv. alfalfae.

(b) P. marginalis pv. marginalis.

Table 2. Differential phenotypic features.

	P. solanacearum (NCPPB 325)	*P. solanacearum* (TCRI 01)	*P. solanacearum* (TCRI 02)	*P. solanacearum* (VOPRI 5)	*P. solanacearum* (VOPRI 26)	% of strains positive	*P. syringae* pv. tabaci	*P. syringae* pv. syringae	*P. syringae* pv. morsprunorum	*P. syringae* pv. mellea	*P. syringae* pv. savastanoi	*P. caricapapayae*	% of strains positive	*P. fluorescens* bv. I	*P. fluorescens* bv. II a	*P. fluorescens* bv. II b	*P. fluorescens* bv. III	*P. tolaasii*	% of strains positive	*P. angulata*	*P. avenae*	No. of strains positive	*P. viridiflava*	*P. aeruginosa*	*P. cepacia*	*P. amygdali*
Production of:																										
Fluorescent pigments	-	-	-	-	-	0	+	+	+	+	+	+	100	+	+	+	+	+	100	+	-	1	+	+	-	-
Xanthomonadins	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-
Urease activity	-	-	-	-	-	0	+	+	+	+	+	+	100	+	+	-	+	+	80	-	-	0	-	-	-	-
Oxidase reaction	+	+	+	+	+	100	-	-	-	-	-	-	0	+	+	+	+	+	100	-	-	0	-	+	+	+
H₂S from cysteine	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	-	+	+	80	+	+	2	+	+	-	-
Asparagine as sole source of C and N	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	+
Protein digestion:																										
"purple milk"	+	-	+	-	-	40	-	+	-	-	-	+	33	-	+	+	+	+	80	+	+	2	+	+	+	+
Milk agar plates	-	-	-	-	-	0	-	-	-	-	-	-	0	-	+	+	+	+	80	-	-	0	-	-	+	+
Nitrate reduction	+	+	+	+	+	100	-	-	-	-	-	-	0	-	+	-	-	-	20	-	+	1	-	-	-	-
Acetoin production	-	-	-	-	-	0	-	+	+	+	-	+	67	-	-	-	-	-	0	-	-	0	-	-	-	-
Oxidative (H & L)	-	+	+	+	+	80	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	-
Arginine dihydrolase	-	-	-	-	-	0	-	-	-	-	-	+	17	+	+	+	+	+	100	+	+	2	+	+	-	-
β-Galactosidase	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-
PHB accumulation	+	+	+	+	+	100	-	-	-	-	-	-	0	-	-	-	-	-	0	-	+	1	-	-	+	-
Hydrolysis of:																										
Gelatin	-	-	-	-	-	0	+	+	+	-	-	+	67	+	+	+	+	+	100	+	+	2	+	+	+	+
Starch	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-
Aesculin	+	-	+	-	-	40	+	+	+	+	+	+	100	+	+	+	+	-	80	-	-	0	+	-	+	-
Tween 80 (esterase)	+	+	+	+	+	100	+	-	+	+	+	+	83	+	+	-	+	+	80	+	+	2	+	+	+	-
Tyrosinase	+	+	+	+	+	100	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-
Acid production on Dye's medium C from:																										
L(+)-arabinose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	+
Cellobiose	-	+	+	+	+	80	-	-	-	-	-	-	0	-	+	-	+	+	60	+	+	2	-	-	+	-
Fructose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	+
Galactose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	-
Glucose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	+
Mannose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	-	+	-
Raffinose	-	-	-	-	-	0	+	+	+	-	-	+	67	+	+	+	-	-	60	+	-	1	+	+	-	+
Melibiose	-	-	-	-	-	0	+	+	+	+	+	+	100	+	+	+	+	+	100	+	-	1	+	+	-	+
Melezitose	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	+
Rhamnose	-	-	-	-	-	0	+	+	+	+	+	-	83	+	+	+	+	+	100	-	-	0	-	-	-	+
Trehalose	+	+	+	+	+	100	-	-	-	-	-	-	0	+	+	+	+	+	100	+	-	1	+	+	+	+
Xylose	+	+	+	+	+	100	+	+	+	+	+	-	83	+	+	+	+	+	100	+	+	2	+	+	+	+
Sucrose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	-	-	60	-	-	0	-	-	+	-
Lactose	-	+	+	+	+	80	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	+	-
Maltose	-	+	+	+	+	80	-	-	-	-	-	-	0	-	+	+	-	+	60	-	+	1	-	-	+	-
Ribose	+	+	+	-	-	60	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	-
meso-Inositol	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	-	1	+	-	+	-
Adonitol	-	-	-	-	-	0	-	-	+	-	-	-	17	+	+	-	+	+	80	-	-	0	-	-	+	-
Dulcitol	-	+	-	-	-	20	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	+	-
Mannitol	-	-	-	-	-	0	+	+	+	+	+	+	100	+	-	-	+	-	40	+	-	1	+	+	+	-
Sorbitol	-	-	-	-	-	0	+	+	+	+	+	+	100	+	+	+	-	+	80	+	+	2	+	+	+	+
Erythritol	-	-	-	-	-	0	+	+	+	+	-	+	83	+	+	+	+	+	100	+	+	2	+	-	-	-
Dextrin	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-
Starch	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	+
Glycerol	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	-
Glycogen	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-

Table 2. (continued).

	PHENON II																PHENON III			
	GROUP I			GROUP 2																
				SUB-GROUP I	SUB-GROUP 2					SUBGROUP 3										
	2 strains			5 strains						6 strains							2 strains			
	X. fragariae	*X. axonopodis*	No. of strains positive	*X. campestris* pv. *campestris*	*X. campestris* pv. *phaseoli*	*X. campestris* pv. *viticola*	*X. campestris* pv. *cassavae*	*X. campestris* pv. *pruni*	% of strains positive	*X. campestris* pv. *manihotis*	*X. c.* pv. *mangiferaeindicae* (NCPPB)	*X. c.* pv. *mangiferaeindicae* (0823)	*X. c.* pv. *mangiferaeindicae* (0836)	*P. mangiferaeindicae* (UP 89)	*X. c.* pv. *mangiferaeindicae* (D42)	% of strains positive	*X. maltophilia*	*P. hibiscicola*	No. of strains positive	*Xy. ampelinus*
Production of:																				
Fluorescent pigments	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Xanthomonadins	+	+	2	+	+	−	+	+	80	−	−	−	−	−	−	0	−	−	0	−
Urease activity	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	+
Oxidase reaction	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
H₂S from cysteine	−	+	1	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	+
Asparagine as sole source of C and N	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	+	+	2	−
Protein digestion:																				
"purple milk"	−	+	1	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	−
Milk agar plates	−	−	0	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	−
Nitrate reduction	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	+	+	2	−
Acetoin production	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Oxidative (H & L)	+	−	1	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	+
Arginine dihydrolase	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
β-Galactosidase	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	+
PHB accumulation	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Hydrolysis of:																				
Gelatin	+	−	1	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	−
Starch	+	+	2	+	+	+	−	−	80	+	−	−	−	−	−	13	−	−	0	−
Aesculin	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	−
Tween 80 (esterase)	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	+
Tyrosinase	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	+	1	−
Acid production on Dye's medium C from:																				
L(+)-arabinose	−	−	0	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	+
Cellobiose	−	−	0	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	−
Fructose	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	−
Galactose	+	−	1	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	+
Glucose	+	+	2	+	+	+	+	+	100	+	−	+	+	+	+	88	+	+	2	−
Mannose	+	−	1	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	−
Raffinose	−	−	0	−	+	+	+	−	60	−	+	+	+	+	+	88	−	−	0	−
Melibiose	−	−	0	+	+	+	−	−	60	+	+	+	+	+	+	100	−	−	0	−
Melezitose	−	−	0	−	−	−	−	−	0	−	+	−	−	+	−	38	−	−	0	−
Rhamnose	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Trehalose	+	+	2	+	+	+	−	+	80	+	+	+	+	+	+	100	−	−	0	−
Xylose	−	−	0	−	−	−	+	+	40	−	−	+	+	+	−	50	−	−	0	−
Sucrose	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	−
Lactose	−	−	0	−	−	−	+	+	40	−	−	−	−	−	−	0	−	−	0	−
Maltose	−	−	0	+	+	+	+	−	80	−	−	−	−	−	−	0	−	−	0	−
Ribose	−	−	0	+	+	+	−	−	60	−	+	+	−	+	+	67	−	−	0	−
meso-Inositol	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Adonitol	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Dulcitol	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Mannitol	−	−	0	+	−	+	−	−	40	−	−	−	−	−	−	0	−	−	0	−
Sorbitol	−	−	0	−	−	−	+	−	20	−	−	−	−	−	−	0	−	−	0	−
Erythritol	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Dextrin	−	−	0	+	+	+	+	−	80	−	−	−	+	−	−	13	+	−	1	+
Starch	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Glycerol	−	−	0	+	+	+	−	+	80	+	+	+	+	+	+	100	−	−	0	+
Glycogen	−	−	0	+	+	−	−	−	40	−	−	−	−	−	−	0	−	−	0	−

corresponding to the description of Xy. ampelinus in Bergey's Manual of Systematic Bacteriology (BRADBURY, 1984). Therefore, only this strain was used for the phenotypic tests. The slow-growing strain of Xy. ampelinus could not be mass-cultured successfully for electrophoresis, most probably due to the standardised broth medium, which lacked the necessary growth factors for Xy. ampelinus, that had to be used throughout. The fast-growing strain of Xy. ampelinus, however, grew satisfactorily in this medium, producing a mucoid, light-yellow type of growth very similar to that of X. axonopodis. For the P. solanacearum strains a preliminary investigation showed no difference in the electrophoretic protein patterns of the different colony types, so that only the white, mucoid type from each strain was used for the phenotypic tests. According to BUDDENHAGEN and KELMAN (1964) the white, fluidal growth is characteristic of the virulent wild type, while the avirulent mutants form small butyrous colonies.

Gram-stain negative, catalase production and motility were the only positive features for all 38 strains used. The following 5 features were negative throughout: production of indole; fermentative reaction using glucose on HUGH and LEIFSON'S O/F medium and acid production from inulin, salicin and α-methyl-D-glucoside.

The results of the tests giving differential reactions are summarised in Table 2. The average reproducibility of tests was 96%.

Numerical analysis of phenotypic features

The phenogram obtained after numerical analysis of 53 unit characters of 24 Pseudomonas and 14 Xanthomonas species and pathovars using the S_{SM} coefficient and the unweighted average pair-group cluster analysis, is shown in Fig. 1. All Pseudomonas and Xanthomonas strains were associated at a level of 75,7% S_{SM}. Three phenons and three solitary strains were differentiated at

Fig. 1. Dendrogram of the similarity coefficient, S_{SM}, grouped by the unweighted average linkage cluster analysis, showing the taxonomic relationships between 24 phytopathogenic <u>Pseudomonas</u> and 14 <u>Xanthomonas</u> spp. and pathovars based on phenotypic features.
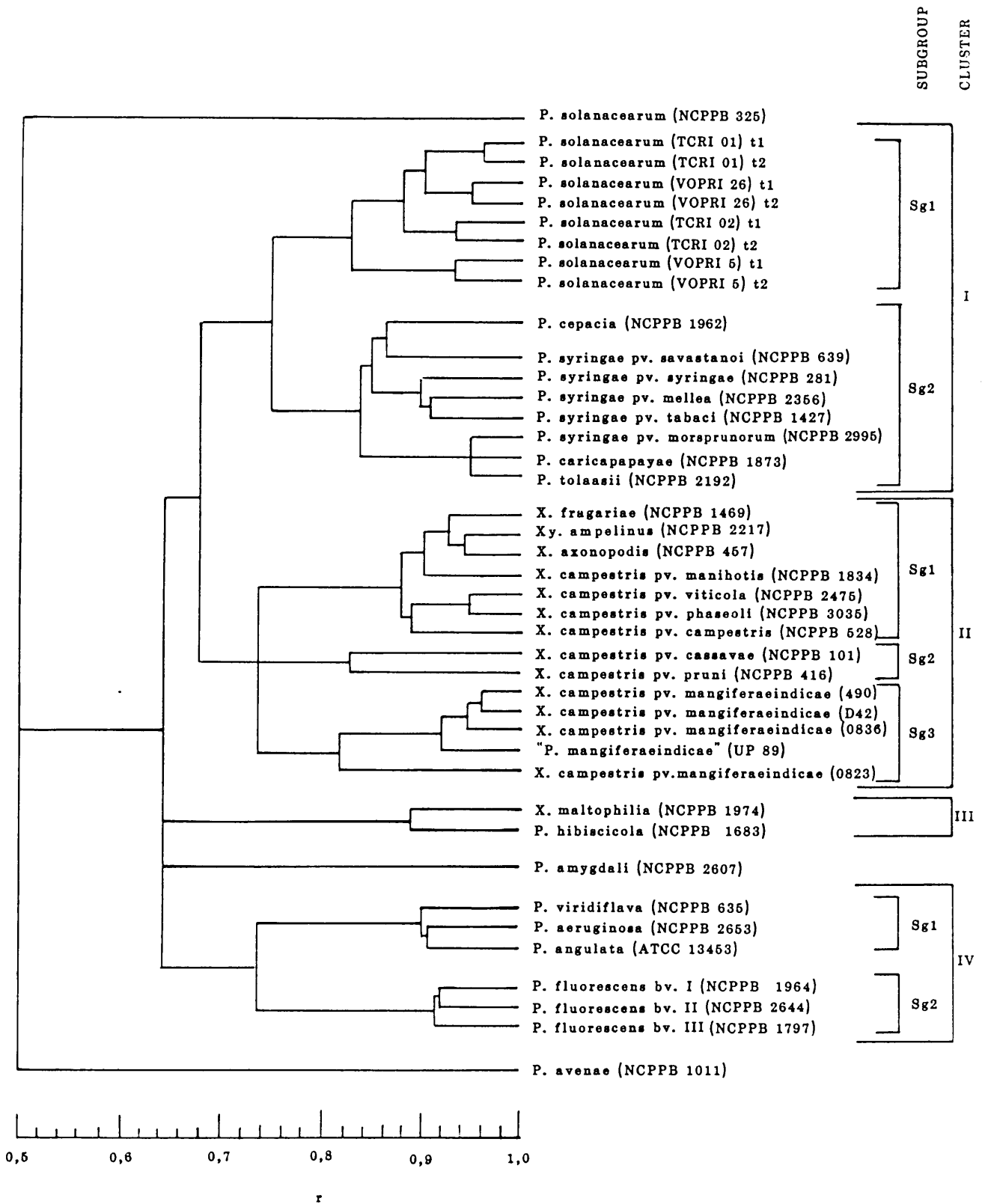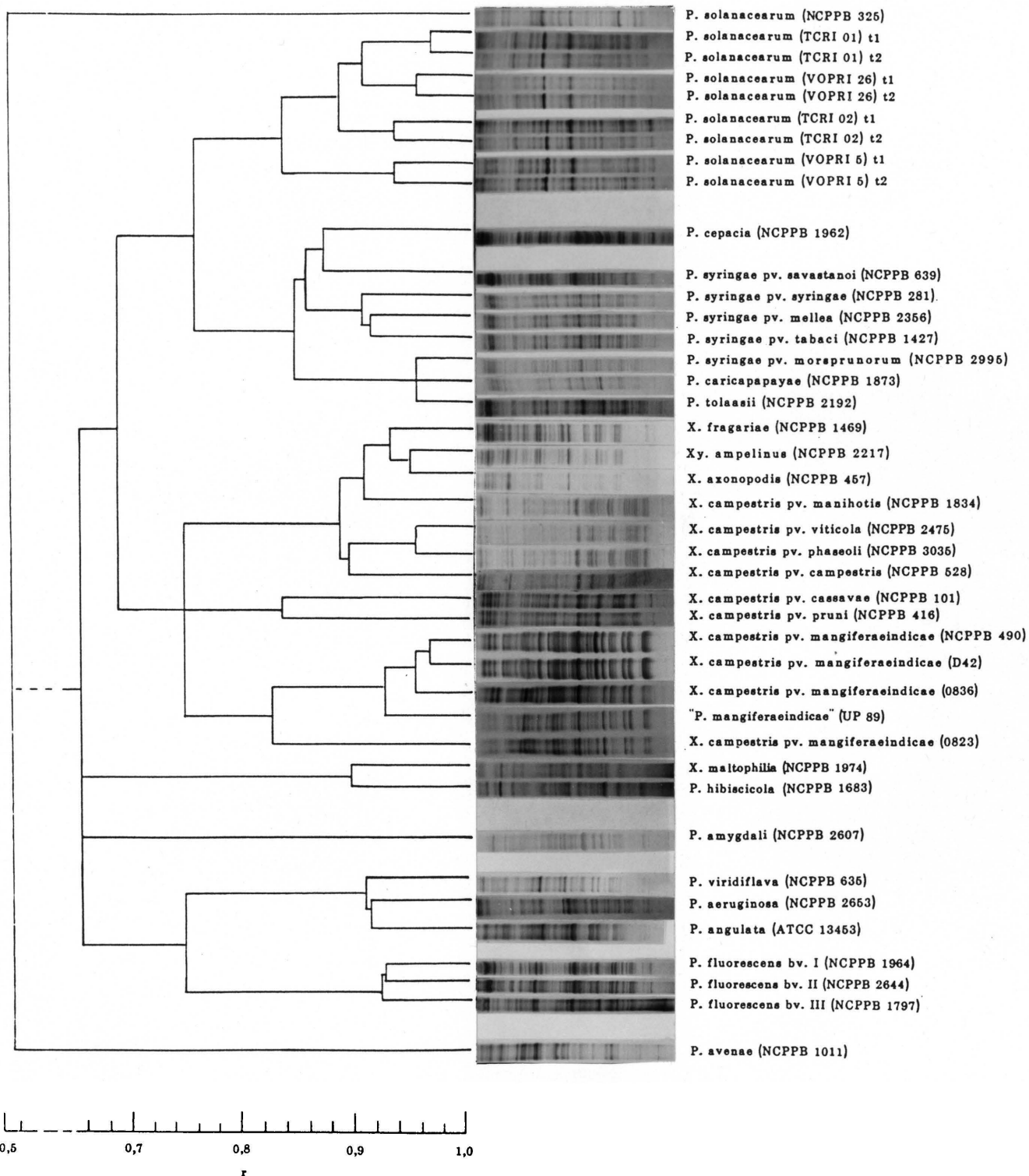
Fig. 1.

SUBGROUP GROUP PHENON

P. solanacearum (NCPPB 325)
P. solanacearum (TCRI 01)
P. solanacearum (VOPRI 26)
P. solanacearum (VOPRI 5)
P. solanacearum (TCRI 02)

G1

P. syringae pv. tabaci (NCPPB 1427)
P. syringae pv. syringae (NCPPB 281)
P. syringae pv. morsprunorum (NCPPB 2995)
P. syringae pv. mellea (NCPPB 2356)
P. syringae pv. savastanoi (NCPPB 639)
P. caricapapayae (NCPPB 1873)

Sg1

P. fluorescens bv. I (NCPPB 1964)
P. fluorescens bv. II (NCPPB 2644)
P. fluorescens bv. III (NCPPB 1797)
P. tolaasii (NCPPB 2192)
P. fluorescens bv. II (NCPPB 667)

Sg2 G2 I

P. angulata (ATCC 13453)
P. avenae (NCPPB 1011)
P. viridiflava (NCPPB 635)
P. aeruginosa (NCPPB 2653)

Sg3

X. fragariae (NCPPB 1469)
X. axonopodis (NCPPB 457)

G1

X. campestris pv. campestris (NCPPB 528)
X. campestris pv. phaseoli (NCPPB 3035)
X. campestris pv. viticola (NCPPB 2475)

Sg1

X. campestris pv. cassavae (NCPPB 101)
X. campestris pv. pruni (NCPPB 416)

Sg2 G2 II

X. campestris pv. mangiferaeindicae (0823)
X. campestris pv. mangiferaeindicae (0836)
X. campestris pv. manihotis (NCPPB 1834)
X. campestris pv. mangiferaeindicae (D42)
"P. mangiferaeindicae" (UP 89)
X. campestris pv. mangiferaeindicae (NCPPB 490)

Sg3

P. cepacia (NCPPB 1962)

Xy. ampelinus (NCPPB 2217)

X. maltophilia (NCPPB 1974)
P. hibiscicola (NCPPB 1683)

III

P. amygdali (NCPPB 2607)

E. coli

B. cereus

60   70   80   90   100

% S_{SM}

84,0% S_{SM}. Twenty of the 24 (83,8%) pseudomonads clustered in phenon I and 13 of the 14 (94,4%) xanthomonads in phenon II.

Two groups were differentiated at 84% S_{SM} in phenon I, namely a P. solanacearum group (G1) and a group with P. fluorescens biovars (G2) as core. The following strains were also assigned to this latter group: P. syringae pathovars (5 strains - united above 90% S_{SM}), P. caricapapayae, P. tolaasii, P. angulata, P. avenae, P. viridiflava and P. aeruginosa.

Phenon II also consisted of two groups, namely a group formed by X. fragariae and X. axonopodis (G1), and a X. campestris group (G2 - 11 strains - united at 90% S_{SM}). Xylophilus ampelinus, a former atypical xanthomonad (PALLERONI, 1984;   WILLEMS et al., 1987), clustered separately.

Phenon III contained the generically misnamed Xanthomonas maltophilia (SWINGS et al., 1983) and its probable synonym P. hibiscicola (an atypical phytopathogenic species) (PALLERONI, 1984) with a similarity of 99% S_{SM} between these two strains. A clustering level of 78% S_{SM} was obtained with the rest of the peseudomonads and the xanthomonads.

P. cepacia and P. amygdali did not cluster in any specific group. The two control organisms, E. coli and B. cereus each clustered separately at a lower level.

Numerical analysis of protein gel electrophoregrams

Reproducibility limits of electrophoresis were above r = 0,95 as determined with three independently grown cultures of the same strain. The most typical electrophoregram of each strain (i.e. the electrophoregram giving the highest correlation value) was used for the final clustering shown in Figs. 2 and 3.

All strains were associated at r = 0,50. At r = 0,74 four electrophoretic clusters were delineated. Cluster I consisted of

Fig. 2. Dendrogram of the correlation coefficient, r, grouped by the unweighted average linkage cluster analysis, showing the taxonomic relationships between 23 phytopathogenic Pseudomonas and 14 Xanthomonas spp. and pathovars based on protein electro-phoretic patterns.

41

Fig. 2.

SUBGROUP CLUSTER

P. solanacearum (NCPPB 325)
P. solanacearum (TCRI 01) t1
P. solanacearum (TCRI 01) t2
P. solanacearum (VOPRI 26) t1
P. solanacearum (VOPRI 26) t2
P. solanacearum (TCRI 02) t1
P. solanacearum (TCRI 02) t2
P. solanacearum (VOPRI 5) t1
P. solanacearum (VOPRI 5) t2
P. cepacia (NCPPB 1962)
P. syringae pv. savastanoi (NCPPB 639)
P. syringae pv. syringae (NCPPB 281)
P. syringae pv. mellea (NCPPB 2356)
P. syringae pv. tabaci (NCPPB 1427)
P. syringae pv. morsprunorum (NCPPB 2995)
P. caricapapayae (NCPPB 1873)
P. tolaasii (NCPPB 2192)
X. fragariae (NCPPB 1469)
Xy. ampelinus (NCPPB 2217)
X. axonopodis (NCPPB 457)
X. campestris pv. manihotis (NCPPB 1834)
X. campestris pv. viticola (NCPPB 2475)
X. campestris pv. phaseoli (NCPPB 3035)
X. campestris pv. campestris (NCPPB 528)
X. campestris pv. cassavae (NCPPB 101)
X. campestris pv. pruni (NCPPB 416)
X. campestris pv. mangiferaeindicae (490)
X. campestris pv. mangiferaeindicae (D42)
X. campestris pv. mangiferaeindicae (0836)
"P. mangiferaeindicae" (UP 89)
X. campestris pv.mangiferaeindicae (0823)
X. maltophilia (NCPPB 1974)
P. hibiscicola (NCPPB 1683)
P. amygdali (NCPPB 2607)
P. viridiflava (NCPPB 635)
P. aeruginosa (NCPPB 2653)
P. angulata (ATCC 13453)
P. fluorescens bv. I (NCPPB 1964)
P. fluorescens bv. II (NCPPB 2644)
P. fluorescens bv. III (NCPPB 1797)
P. avenae (NCPPB 1011)

Sg1 Sg2 I
Sg1 Sg2 Sg3 II
III
Sg1 Sg2 IV

0,5 0,6 0,7 0,8 0,9 1,0

r

Fig. 3. Dendrogram showing the protein electrophoregrams of the 37 phytopathogenic <u>Pseudomonas</u> and <u>Xanthomonas</u> species and pathovars.

Fig. 3.

P. solanacearum (NCPPB 325)
P. solanacearum (TCRI 01) t1
P. solanacearum (TCRI 01) t2
P. solanacearum (VOPRI 26) t1
P. solanacearum (VOPRI 26) t2
P. solanacearum (TCRI 02) t1
P. solanacearum (TCRI 02) t2
P. solanacearum (VOPRI 5) t1
P. solanacearum (VOPRI 5) t2

P. cepacia (NCPPB 1962)

P. syringae pv. savastanoi (NCPPB 639)
P. syringae pv. syringae (NCPPB 281)
P. syringae pv. mellea (NCPPB 2356)
P. syringae pv. tabaci (NCPPB 1427)
P. syringae pv. morsprunorum (NCPPB 2995)
P. caricapapayae (NCPPB 1873)
P. tolaasii (NCPPB 2192)
X. fragariae (NCPPB 1469)
Xy. ampelinus (NCPPB 2217)
X. axonopodis (NCPPB 457)
X. campestris pv. manihotis (NCPPB 1834)
X. campestris pv. viticola (NCPPB 2475)
X. campestris pv. phaseoli (NCPPB 3035)
X. campestris pv. campestris (NCPPB 528)
X. campestris pv. cassavae (NCPPB 101)
X. campestris pv. pruni (NCPPB 416)
X. campestris pv. mangiferaeindicae (NCPPB 490)
X. campestris pv. mangiferaeindicae (D42)
X. campestris pv. mangiferaeindicae (0836)
"P. mangiferaeindicae" (UP 89)
X. campestris pv. mangiferaeindicae (0823)
X. maltophilia (NCPPB 1974)
P. hibiscicola (NCPPB 1683)

P. amygdali (NCPPB 2607)

P. viridiflava (NCPPB 635)
P. aeruginosa (NCPPB 2653)
P. angulata (ATCC 13453)
P. fluorescens bv. I (NCPPB 1964)
P. fluorescens bv. II (NCPPB 2644)
P. fluorescens bv. III (NCPPB 1797)

P. avenae (NCPPB 1011)

0,5   0,7   0,8   0,9   1,0

r

two subgroups corresponding to the P. solanacearum group (Sg1) and the P. syringae group (Sg2) respectively. Included in this latter group were P. caricapapayae and P. tolaasii at r = 0,95 and P. cepacia at r = 0,87.

Cluster II comprised all the Xanthomonas strains (except X. maltophilia). Three equidistant subgroups could be delineated at r = 0,74.

Cluster III consisted of the 2 strains X. maltophilia and P. hibiscicola, clustering at r = 0,89 with each other, and r = 0,68 with the rest of the strains.

Cluster IV included two subgroups consisting of P. viridiflava, P. aeruginosa and P. angulata clustering at r = 0,90 in the one subgroup (Sg1) and the 3 P. fluorescens biovars united at r = 0,91 in the other (Sg2).

The reference strain of P. solanacearum (NCPPB 325), P. amygdali and P. avenae each clustered separately.

DISCUSSION

Morphological, physiological and biochemical features

Most of the results from the phenotypic tests correspond to those in the literature (BRADBURY, 1984; PALLERONI, 1984). A few exceptions are nitrate reduction in the case of P. aeruginosa; hydrolysis of Tween 80 for P. amygdali; and acid production from erythritol for X. maltophilia, from lactose for P. solanacearum (TCRI 02) and P. cepacia, from fructose for X. axonopodis and from galactose for X. fragariae. In some cases, especially in the Pseudomonas group, contradictory results were obtained for protein digestion tested in "purple milk" and on milk agar plates.

Fourteen of the 24 phytopathogenic Pseudomonas species were characterised by the production of a fluorescent pigment, while 6 of the 14 xanthomonads characteristically produced xanthomonadins. Eighteen of the 38 (47,4%) strains could therefore not be differentiated by any one of these characteristic features.

The following two features were useful in differentiating the pseudomonads from the xanthomonads in all instances (phenon III excluded): β-galactosidase reaction and the utilisation of asparagine as sole source of C and N.

Uniformly positive results for the pseudomonads (phenon III excluded) were obtained from asparagine as sole source of C and N, and acid production from L(+)-arabinose, fructose and glucose. Uniformly negative results were obtained for the β-galactosidase reaction, hydrolysis of starch and acid production from melezitose, dextrin and glycogen. Tyrosinase activity differentiated the P. solanacearum subgroup from the rest of the pseudomonads, but not one feature could be found that could differentiate either the P. syringae or the P. fluorescens subgroups as a whole from the rest of the pseudomonads.

Uniformly positive results for the xanthomonads (X. maltophilia excluded) were obtained for the β-galactosidase and esterase (hydrolysis of Tween 80) reactions. The following features were also uniformly positive: hydrolysis of aesculin , and acid production from fructose and sucrose. The xanthomonads were uniformly negative for the utilisation of asparagine as sole source of C and N; oxidase reaction; nitrate reduction; arginine dihydrolase; poly-β-hydroxybutyrate accumulation; tyrosinase reaction; and acid production from rhamnose, meso-inositol, adonitol, dulcitol, erythritol and starch. Acid production from L(+)-arabinose and cellobiose were the only features differentiating the X. fragariae - X. axonopodis group from the X. campestris group.

The following features were non-differential (i.e. both positive and negative results in both genera): urease activity; H_2S from cysteine; protein digestion; oxidative reaction on glucose; hydrolysis of gelatin and aesculin; and acid production from galactose, mannose, raffinose, melibiose, trehalose, xylose, lactose, maltose, ribose, mannitol, sorbitol and glycerol.

The problem of differentiating between pathovars by means of biochemical tests, is illustrated by the P. syringae and X. campestris groups for which, in most cases, all the features were either positive or negative respectively for all strains tested. This resulted in a >90% S_{SM} clustering level for these individual pathovar groups, indicating that they share 90% or more of their features, and therefore only a few phenotypic features were useful for differentiation.

For many plant pathologists, these conventional cultural, biochemical and physiological tests are the only available means of identification. The following practical problems were, however, experienced during this study: Large quantities of and many different media were required and the processes of the preparation and inoculation thereof were tedious and time-consuming. Several tests also failed to distinguish between closely related organisms, mainly because they bear no relation to the plant habitat of the pathogens (e.g. gelatin lique= faction). Results were often delayed, as in the case of the xanthomonads where results of acid production from carbon sources still varied up to 42 d. Colour changes as indicated by means of pH indicators, which form the basis of many biochemical tests, were sometimes transitory or so slight as to be virtually impossible to read or to interpret objectively. The utilisation of asparagine as sole source of both C and N, and β-galactosidase activity were the only features tested that differentiated the pseudomonads from the xanthomonads. Although no difficulties were experienced with the ONPG test (β-galactosidase), extreme care had to be taken with the asparagine test to ensure

ultraclean glassware and to avoid subjectivity in the case of weak growth. Growth conditions and the cell concentrations of inocula had therefore to be rigorously controlled, with double checking of weak growth. SNEATH (1986) furthermore found that microbiological data are prone to more experimental error than is commonly realised, causing the average difference in replicate tests on the same strain commonly to be as high as 5%.

Numerical analysis of phenotypic features

The results of the numerical analysis of the phenotypic features correspond to a high degree with the present taxonomic situation in these genera:

The three rRNA homology groups, groups I, II and V (PALLERONI, 1984) are clearly reflected in the phenogram (Fig. 1). Group 2 of phenon I corresponds with rRNA group I. Also assigned to this group are P. caricapapayae, P. tolaasii and P. avenae, three of the species of section V of Pseudomonas (PALLERONI, 1984) of which the natural relationships with other well characterised species of the genus are largely unknown. The fourth species of this group, P. amygdali, however, clustered separately. P. caricapapayae and P. tolaasii, both being fluorescent organisms, thus clustered with the other fluorescent pseudomonads, whereas P. amygdali, a non-fluorescent member of section V clustered separately. This classification of P. caricapapayae and P. tolaasii supports the findings of DE VOS et al. (1985) who placed these two organisms in the P. fluorescens rRNA branch. According to these authors P. amygdali, however, belongs to the same branch. P. avenae, on the other hand, was found to belong to the "P". acidovorans rRNA branch (rRNA group III) (DE VOS et al., 1985), while it clustered in group 2 of phenon I (rRNA group I) of the phenogram.

All the P. solanacearum strains clustered in a separate group, corresponding to rRNA homology group II (PALLERONI, 1984). P. cepacia, however, did not cluster in this group as was suggested

by literature (PALLERONI, 1984; DE VOS et al., 1985). Furthermore, the one P. solanacearum strain from potatoes (VOPRI 26) was more closely related to the P. solanacearum strain from tobacco (TCRI 01) than to the other potato isolate of P. solanacearum (VOPRI 5), thus not reflecting any correlation between host-specificity and phenotypic features in this group.

Except for X. maltophilia, the xanthomonads clustered in a single phenon. All the X. campestris pathovars grouped together, while the other two Xanthomonas species clustered separately. X. campestris pv. manihotis (a nonpigmented pathovar) clustered in the core of the nonpigmented X. campestris pv. mangiferaeindicae group. Xy. ampelinus was excluded from this cluster in accordance with results that this is an atypical xanthomonad (PALLERONI, 1984; WILLEMS et al., 1987).

The high relatedness of X. maltophilia and P. hibiscicola (99% S_{SM}) supports the view that they might be synonyms (PALLERONI, 1984). They did, however, not cluster as close to the Xanthomonas group as suggested by PALLERONI et al. (1973), SWINGS et al. (1983) and DE VOS et al. (1985). They were in fact less related to the Xanthomonas group than the majority of the Pseudomonas strains studied.

According to AUSTIN and PRIEST (1986) it is generally accepted that "species" should be defined at the 80-85% similarity level if using S_{SM}-UPGMA generated dendrograms. All strains that coalesce above this level may be considered presumptive members of a species. Thirty-five of the 38 strains used in this phenotypic study clustered above this species level. The cause of these high similarity values may be traced back to the relatively small number of tests used (53), but again illustrates the problem with which this study is concerned: the strains in this group are too closely related to be differentiated at a satisfactory level and in a relatively short time by a manageable amount of phenotypic tests.

49

Standardised electrophoretic procedures

Although it cannot be denied that a certain degree of technical expertise is required when using this technique, the advantages and possibilities of electrophoresis cannot be overemphasised. As several variations of the electrophoretic technique exist, a standardised procedure, as the one described here, will enhance the reproducibility and enable the accurate comparison of results between different phytobacterial laboratories.

The advantages of using a whole cell extract are obvious. The manipulations are minimal and within a few hours the electrophoresis is completed. More protein bands are also obtained than with purified fractions, which is an advantage for numerical analysis. Sonication proved to be the method of choice for preparing the protein extract as it is relatively fast in comparison with the French Pressure Cell, for example, and ensures minimal oxidation of the sample.

Freshly prepared samples yield excellent, reproducible results with optimal resolution. In sample solutions frozen for more than approximately 6 weeks, some bands are lost or do not stain as intense as in freshly prepared samples, while the resolution also deteriorates.

With the highly efficient and simplified electrophoretic apparatus presently available, negligible difficulty is experienced with the assembling and casting of gels. Slab gels are preferred to rod gels because of several advantages (HAMES, 1981). The most important advantage is that as many as 25 samples can be electrophoresed under identical conditions in a single gel such that the band patterns produced are directly comparable. This also improves the reproducibility, in contrast to rod gels where differences in the polymerization reactions may occur between different gels of the same batch, thus hampering accurate comparison of protein patterns (Prof. Stegemann,

personal communication). In most of the modern apparatus, up to 12 gels can be run simultaneously, thus ensuring identical conditions for the electrophoresis of many samples. In this case, however, care has to be taken to prevent too much diffusion of loaded samples prior to electrophoresis because of the extended loading time required for so many gels. Other advantages of slab gels are the shorter time required for the preparation of gels, the more effective dissipation of heat produced during electrophoresis and the smaller risk of optical artifacts during spectrophotometry and photography of gels (HAMES, 1981).

Various authors have reported on the powerful resolution, sensitivity and reproducibility attainable using the discontinuous SDS-PAGE system of LAEMMLI (1970) (AMES et al., 1974; HAMES, 1981). Discontinuous buffer systems employ different buffer ions in the gel compared with those in the electrode reservoirs. Most discontinuous buffer systems, like the present one, have discontinuities of both buffer composition and pH (HAMES, 1981), and consist of two gel layers, namely a small pore separating or resolving gel (pH 8,8) and a large-pore stacking gel (pH 6,8). The major advantage of discontinuous buffer systems over continuous buffer systems is the relatively large volumes of dilute protein samples that can be applied to gels with no loss of resolution. This is due to the concentration of samples into narrow zones during migration through the stacking gel prior to their separation in the resolving gel, resulting in improved band resolution (HAMES, 1981).

In this system, the ionic detergent, SDS, is used to dissociate all proteins into their individual polypeptide subunits. What we therefore actually have in SDS-PAGE is protomer or sub-unit sizing (Prof. Stegemann, personal communication), in contrast to PAGE using nondissociating buffers where the whole protein molecule with all its subunits stays intact, thus also preserving biological activity. In this

instance separation is according to size as well as charge density. In the case of SDS-PAGE, the SDS-polypeptide complexes have essentially identical charge densities as one molecule of SDS binds per amino acid, giving each protein molecule the same charge per amino acid residue (FREIFELDER, 1985). The intrinsic charges of the polypeptide are insignificant compared to the negative charges of the bound detergent. The protomers or sub-units are therefore separated in the resolving gel strictly according to polypeptide size (HAMES, 1981). Mercaptoethanol reduces disulphide bonds, allowing proteins to bind considerably increased amounts of SDS (GORDON, 1975). This denaturing process is enhanced by high temperatures (95 to 100°C), hence the heating of the sonicated samples prior to electrophoresis.

Removing the insoluble cellular debris by centrifugation prior to electrophoresis proved valuable, as it eliminated protein streaking along gel tracks. Overloading of the gel resulted in distorted bands and for this reason standardisation of the amount of sample applied to gels was essential. Effective cooling of the gel during electrophoresis was very important as it reduced the "smile" effect which hampered normalization of the electrophoregrams.

Total separation of the protein samples, without exclusion of high molecular mass proteins at the top of the gel, was obtained using a polyacrylamide resolving gel of final monomer concentration (%T) = 10,0% and crosslinking monomer concentration in the stock solution (%C) = 2,67%. It needs to be stressed that, in order to produce the same set of proteins, a bacterial strain has to be grown under standardised conditions. For optimal reproducibility, rigorously controlled and standardised procedures for the cultivation of cells, preparation of extracts and electrophoresis of the proteins had to be followed. Chemicals specially purified for electrophoresis also enhanced resolution and reproducibility.

Normalization and numerical analysis of protein electrophoregrams

The value of electrophoretic data depends upon the procedure and methods used in interpreting the results (SHIPTON and FLEISCHMANN, 1969). As the eye and human brain are unable to compare and remember large numbers of protein profiles, computer-assisted analysis is essential (KERSTERS and DE LEY, 1980). While visual inspection detects only qualitative similarities between electrophoregrams, quantitative numerical comparisons are totally objective and might detect unsuspected similarities or differences by a variety of clustering techniques.

The use of internal standards is essential for reproducible results between different gels. Inclusion of the standards in the sample tracks was, however, unsatisfactory, as thyroglobulin separated into 2 major and 4 minor bands (confirmed by Sigma - Catalogue of biochemical and organic compounds, 1985) which masked some of the sample bands. The use of lysozyme simplified accurate superimposing of standards and samples during normalization.

This method of dividing the scans into a fixed number of positions with the aid of reference proteins, and measuring the heights, irrespectively of the position being on a peak or part of a valley, is to my opinion much more objective than relying on R_m (relative mobility) values. For R_m values certain bands are visually (and perhaps subjectively) selected for comparisons and when optimal resolution is not obtained, it is often difficult to decide visually precisely where to read the position of a band. The first method therefore incorporates both similarities and dissimilarities on a consistent and reproducible basis.

The only problem encountered when using this system, was the sensitivity of the spectrophotometer. The spectrophotometer used was not sensitive enough to differentiate between narrow bands very close to each other, as is often found at the top of the

gel. Bands such as these were then merely expressed as a single peak with area the sumtotal of the respective areas of the bands involved. This, however, occurred consistently and did not have a noticeable effect on the reproducibility nor the reliability of this metod of analysis. As is stressed by KERSTERS and DE LEY (1975) it is, however, also important to group the photographs or dried electrophoregrams according to the computer results and to inspect them visually for abnormal inclusions in the dendrogram.

SNEATH and SOKAL (1973) and SNEATH (1986), among others, have reported on the advantages and applications of numerical taxonomy in general. Correlation coefficients were most suitable for this numerical analysis, as they are insensitive to the experimental differences in amount of protein applied to gels (KERSTERS and DE LEY, 1975). Average-linkage cluster analysis is most commonly used for taxonomical purposes (SNEATH, 1986), and it can be shown theoretically from the cophenetic correlation that average-linkage cluster analysis gives the most accurate representation of the taxonomic structure (AUSTIN and PRIEST, 1986). For general purposes such as this, a dendrogram is the most useful representation, as the operational taxonomic units (strains) are divided into convenient groups that can be recognized easily.

The taxonomic structure of this group of strains as determined by numerical analysis of their electrophoretic protein patterns

Whereas the overall grouping obtained by using the phenotypic features and the similarity coefficient corresponds to the rRNA homology groups as suggested by PALLERONI (1984), the overall grouping obtained by the numerical analysis of electrophoregrams corresponds more to the separate DNA homology groups suggested by the same author. However, an excellent agreement is found between the separate subgroupings obtained by numerical analysis of phenotypic features and of protein electrophoregrams. Sub-groupings almost identical in both dendrograms are: a P. solanacearum subgroup; a P. syringae subgroup; a P. fluorescens subgroup; a X. fragariae subgroup; a X. campestris pv.

campestris subgroup; a X. campestris pv. cassavae subgroup; a
X. campestris pv. mangiferaeindicae subgroup and a X. maltophilia
subgroup.

The P. solanacearum reference strain (NCPPB 325) did not
cluster with the local P. solanacearum strains, accentuating the
wide strain variation of this pathogen (GARRETT, 1982). This
reference strain clustered totally separately at the low
correlation level of r = 0,50. P. avenae also joined the rest of
the strains at this low correlation level. This is in accordance
with the results of DE VOS et al. (1985) who concluded that the
P. solanacearum branch and the "P". acidovorans branch (of which
P. avenae is a member) should be removed from the genus
Pseudomonas. WILLEMS et al. (1987) also postulated that each
subbranch in the acidovorans rRNA complex deserves generic rank.

For the local P. solanacearum strains a very high correlation
(r > 0,92) was found between the two different colony types
(virulent and avirulent) from each strain. The two different
colony types from each strain clustered with each other first,
before clustering with the other P. solanacearum strains. Again,
as was the case with the phenotypic features, no correlation was
found between host of isolation and electrophoretic clustering or
virulence and electrophoretic clustering.

P. caricapapayae and P. tolaasii were satisfactorily placed in
the P. syringae group, while the inclusion of P. cepacia in this
group was unexpected. In the P. syringae group the two pathovars
from the same host (tobacco), P. syringae pv. mellea and P.
syringae pv. tabaci, clustered at r = 0,91, thus suggesting a
possible correlation between host-specificity and protein
profiles in this group. The separate clustering of the P.
syringae subgroup and the P. fluorescens cluster may be traced
back to their phytopathogenic capabilities. In contrast to the
P. syringae subgroup, consisting only of phytopathogens, P.
fluorescens and P. aeruginosa are considered non-phytopathogenic
members of the P. fluorescens rRNA branch (DE VOS et al., 1985),

while P. marginalis (P. fluorescens bv. II) and P. viridiflava are also not yet fully specialised as phytopathogenic species, resembling the saprophytic species in this rRNA branch (PALLERONI, 1984). Electrophoresis therefore differentiated between members of the P. fluorescens rRNA branch on the basis of their phytopathogenic abilities.

The xanthomonads formed a single cluster, clearly differentiated from the pseudomonads. Three subgroups were, however, delineated. All the X. campestris pv. mangiferaeindicae strains clustered in a single subgroup, while the other X. campestris pathovars were divided into the same two subgroups as in the case of the phenotypic features. Internal subdivision of the X. campestris group was also reported by other authors (VAUTERIN et al., 1990). The low levels of DNA homology reported among X. campestris pv. manihotis, X. campestris pv. cassavae and X. campestris pv. campestris (VAN DEN MOOTER et al., 1987), was confirmed by their grouping in this study. X. fragariae and X. axonopodis were more closely related to the first X. campestris subgroup as was reflected by phenotypic features. Xy. ampelinus was also included in this group. As explained previously, this culture did not correspond to the accepted description of this species, and the culture used for electrophoresis might therefore not have been representative of the species. No correlation was found between virulence and protein electrophoretic (or pheno-typic) clustering in the case of the avirulent X. campestris pv. mangiferaeindicae strains used. UP 89 ("P. mangiferaeindicae") clustered tightly with the X. campestris pv. mangiferaeindicae strains, which in turn clustered with the other X. campestris pathovars, justifying its reclassification as X. campestris pv. mangiferaeindicae (ROBBS et al., 1974).

X. maltophilia and P. hibiscicola again formed a separate cluster, supporting the findings of PALLERONI (1984) that there may be a close relationship between them. Again, as in the phenogram, the X. maltophilia group did not cluster significantly close to the Xanthomonas group. Supported by the electrophoretic

(and phenotypic) results, I find PALLERONI's (1984) "more rational" suggestion that a new genus should be created for the former P. maltophilia, which together with Xanthomonas, could constitute a separate new taxon, more acceptable than the inclusion of "P". maltophilia in the genus Xanthomonas (SWINGS et al., 1983). The latter reclassification is questionable when it is considered that the DNA homologies between X. maltophilia strains and Xanthomonas were found to be low, while DNA-rRNA hibridizations placed X. maltophilia ($T_{m(e)}$ range 76,5-78,0°C) at the lower edge of Xanthomonas ($T_{m(e)}$ range 78,8-81,5°C) (SWINGS et al., 1983). While a S_{SM} value of 75% similarity between P. maltophilia and Xanthomonas was calculated (SWINGS et al., 1983), the corresponding S_{SM} value for the pseudomonads was unfortunately not reported. Four important criteria in the present generic definition of the xanthomonads, namely plant pathogenicity, xanthomonadin production, a single polar flagellum and the inability to reduce nitrates, are also not met by X. maltophilia (SWINGS et al., 1983).

As with the phenotypic features, P. amygdali clustered as a solitary species, justifying a more extensive study of this atypical species.

In cluster IV the P. fluorescens biovars formed a tight group, while the rest of the cluster consisted of the other fluorescent pseudomonads, namely P. aeruginosa, P. viridiflava and P. angulata. The inclusion of the latter strain in this group demands attention. P. angulata (FROMME and MURRAY, 1919) STEVENS (1925) was unaccountably omitted from the 8th edition of Bergey's Manual of Determinative Bacteriology (DOUDOROFF and PALLERONI, 1974). It was not even mentioned in one of the four addenda of incompletely described Pseudomonas spp. For this reason it was also omitted from the Approved Lists of Bacterial Names (SKERMAN et al., 1980), although DYE et al. (1975) included P. angulata in their list of species which they proposed should be retained in general use as they had been validly published, while cultures were available.

In the past BRAUN (1937), HILDEBRAND (1971) and AKEHURST (1981) have considered this species to be identical with "P. tabaci" (P. syringae pv. tabaci (WOLF and FOSTER, 1917) YOUNG et al., 1978), although different disease symptoms are produced on their common host, tobacco. This is probably due to the lack of toxin in the case of P. angulata which causes angular leaf spot of tobacco while P. syrinage pv. tabaci causes tobacco wildfire (AKEHURST, 1981).

In the present study, neither the phenotypic features nor the electrophoretic data provided any proof of this alleged relationship between P. angulata and P. syringae pv. tabaci, although the phenotypic results obtained for P. angulata and P. tabaci correspond very well with those described in the literature (BURKHOLDER, 1957).

In both our approaches P. angulata was found to be closely related to P. aeruginosa. One of the P. angulata strains (no. 46) studied by SANDS et al. (1970), also clustered in the P. aeruginosa - P. fluorescens group. Interestingly enough, there are reports of P. aeruginosa associated with tobacco (PALLERONI, 1984), although it is regarded as a low-grade phytopathogen which seldomly attacks plants (SCHROTH et al., 1981). This was a result of CLARA's description in 1930 of a bacterium "Phytomonas polycolor" isolated from a bacterial leaf spot of tobacco which was not wildfire. In 1942 STARR and BURKHOLDER found P. polycolor to be very similar to P. fluorescens, but the 7th edition of Bergey's Manual of Determinative Bacteriology (BURKHOLDER, 1957) accepted the findings of ELROD and BRAUN (1942) who regarded P. polycolor identical to P. aeruginosa. PALLERONI (1984) also support this view. This connection of P. aeruginosa with a tobacco disease was confirmed by the present study with a correlation of r = 0,91 between the electrophoretic protein profiles of the tobacco pathogen P. angulata and P. aeruginosa.

Although P. angulata was omitted from the Approved Lists of Bacterial Names (SKERMAN et al., 1980), it is still recognized as the causal organism of angular leaf spot of tobacco which is of worldwide distribution and which can range from a minor problem to the cause of a severe epidemic (AKEHURST, 1981). This is a potentially dangerous and confusing situation, as names omitted from the Approved Lists are available for re-use for newly discovered bacteria. Because of the similarity in the disease symptoms caused by P. angulata and P. syringae pv. tabaci, they are also still regarded as closely related (AKEHURST, 1981). It is therefore concluded that a thorough comparative study of the phenotypic properties and pathological differences of the strains concerned will be necessary to resolve the uncertainty surrounding the exact identity of P. angulata and to establish whether the omission of P. angulata from the Approved Lists of Bacterial Names was justified.

As a result of this study, the author came to the following conclusions:

According to numerical analysis of phenotypic features and electrophoretic protein patterns a close relationship between the phytopathogenic pseudomonads and the xanthomonads does exist.

SDS-PAGE of the total soluble proteins of the bacterial cell is a powerful, rapid and relatively easy method for the differentiation of phytopathogenic Pseudomonas and Xanthomonas species that can be applicable to many diagnostic plant pathology laboratories.

This technique also provides an unchallenged practical method for the rapid differentiation of pathovars of these two genera that were previously differentiated primarily on the basis of their hosts of isolation.

With the aid of a data bank of reference strains of these two genera, identification of new Pseudomonas or Xanthomonas isolates becomes a trivial procedure, especially in the case of strains of unknown origin, as visual comparison of electrophoregrams alone is sufficient in many cases, thus eliminating time-consuming procedures such as the inoculation of possible hosts or the performance of a great number of phenotypic tests.

Although too few strains from the same hosts were studied to come to a definite conclusion, we are convinced that PAGE, used in collaboration with a data bank, has the potential to be the method so sought after by plant pathologists to reflect the high degree of host specificity shown by closely related organisms.

PAGE can be of invaluable assistance in conventional identification procedures if used to screen for identical isolates or to group great numbers of isolates before identification procedures are undertaken.

PAGE of cellular proteins proved to be a reliable and objective method of great precision of which the results correlate to a great extent with those obtained from phenotypic features, as well as DNA homology and DNA-rRNA homology studies.

Based on the electrophoretic results, the following taxonomic proposals are made:

- P. caricapapayae and P. tolaasii should be included in the P. syringae group as pathovars of P. syringae.

- A new genus should be created for X. maltophilia. P. hibiscicola should be included in this genus as a species closely related to P. maltophilia.

- <u>P</u>. <u>avenae</u> should be generically reclassified.

- <u>P</u>. <u>amygdali</u> is an atypical pseudomonad and its
  taxonomic position should be reconsidered.

- <u>P</u>. <u>angulata</u> is probably a valid nomenspecies and should
  be re-examined to evaluate its omission from the Approved
  Lists of Bacterial Names. We suggest that this name should
  be revived.

The aim of this study was not to solve all the problems in
these two genera, but to accentuate the applicability of PAGE, as
a taxonomic tool, in everyday phytobacterial practices, as it has
been underestimated and neglected in this field for too long.
Most of the taxonomic proposals are therefore mere suggestions
which have to be followed up by more extensive study before
definite taxonomic conclusions can be drawn.

REFERENCES

AKEHURST, B.C.: Tobacco (D. RHIND, G. WRIGLEY, eds.), 2nd ed. London, Longman 1981

ALVAREZ, A.M., BENEDICT, A.A., MIZUMOTO, C.Y.: Identification of xanthomonads and grouping of strains of Xanthomonas campestris pv. campestris with monoclonal antibodies. Phytopathology 75, 722-728 (1985)

AMES, G.F.: Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. biol. Chem. 249, 634-644 (1974)

ANDERSON, N.G., ANDERSON, N.L.: High resolution two-dimensional electrophoresis of human plasma proteins. Proc. Nat. Acad. Sci. USA. 74, 5421 (1977)

AUSTIN, B., PRIEST, F.G.: Modern bacterial taxonomy. Workingham, Van Nostrand Reinold 1986

BILLING, E., GARRETT, C.M.E.: Phages in the identification of plant pathogenic bacteria, pp. 319-338. In: Microbiological classification and identification (M. GOODFELLOW, R.G. BOARD, eds.). London, Academic Press 1980

BRADBURY, J.F.: Genus II. Xanthomonas, pp. 199-210. In: Bergey's manual of systematic bacteriology (N.R. KRIEG, J.G. HOLT, eds.), 9th ed., vol. 1. Baltimore, Williams and Wilkens 1984

BRAUN, A.C.: A comparative study of Bacterium tabacum Wolf and Foster and Bacterium angulatum Fromme and Murray. Phytopathology 27, 283-303 (1937)

BUDDENHAGEN, I., KELMAN, A.: Biological and physiological aspects of bacterial wilt caused by Pseudomonas solanacearum. Ann. Rev. Phytopathol. 2, 203-230 (1964)

BURKHOLDER, W.H.: Genus I. Pseudomonas Migula, 1894. In: Bergey's manual of determinative bacteriology (E.G.D. MURRAY, N.R. SMITH, eds.), 7th ed. London, Bailliere, Tindall and Cox 1957

BURKHOLDER, W.H., STARR, M.P.: The generic and specific characters of phytopathogenic species of Pseudomonas and Xanthomonas. Phytopathology 38, 494-502 (1948)

CLARA, F.M.: A new bacterial leaf disease of tobacco in the Philippines. Phytopathology 20, 691-706 (1930)

COLWELL, R.R., LISTON, J.: Taxonomic analysis with the electronic computer of some Xanthomonas and Pseudomonas species. J. Bact. 82, 913-919 (1961)

DE LEY, J., FRIEDMAN, S.: Similarity of Xanthomonas and Pseudomonas deoxyribonucleic acid. J. Bact. 89, 1306-1309 (1965)

DE LEY, J., PARK, I.W., TIJTGAT, R., VAN ERMENGEM, J.: DNA homology and taxonomy of Pseudomonas and Xanthomonas. J. gen. Microbiol. 42, 43-56 (1966)

DE VOS, P., DE LEY, J.: Intra- and intergeneric similarities of Pseudomonas and Xanthomonas ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 33, 487-509 (1983)

DE VOS, P., GOOR, M., GILLIS, M., DE LEY, J.: Ribosomal ribonucleic acid cistron similarities of phytopathogenic Pseudomonas species. Int. J. system. Bact. 35, 169-184 (1985)

DOUDOROFF, M., PALLERONI, N.J.: Genus I. Pseudomonas Migula 1894, 237 Nom. cons. Opin. 5, Jud. Comm. 1952, In: Bergey's manual of determinative bacteriology (R.E. BUCHANAN, N.E. GIBBONS, eds.), 8th ed. Baltimore, Williams and Wilkins 1974

DOWSON, W.J.: On the systematic position and generic names of the gram-negative bacterial plant pathogens. Zentr. Bakteriol. Parasitenk. Abt.II 100, 177-193 (1939)

DYE, D.W.: The inadequecy of the usual determinative tests for the identification of Xanthomonas spp. N.Z. J. Sci. 5, 393-416 (1962)

DYE, D.W.: Xanthomonas, pp. 45-49. In: Laboratory guide for identification of plant pathogenic bacteria (N.W. SCHAAD, ed.). Minnesota, Am. Phytopath. Soc. 1980

DYE, D.W., BRADBURY, J.F., DICKEY, R.S., GOTO, M., HALE, C.N., HAYWARD, A.C., KELMAN, A., LELLIOTT, R.A., PATEL, P.N., SANDS, D.C., SCHROTH, M.N., WATSON, D.R.W., YOUNG, J.M.: Proposals for a reappraisal of the status of the names of plant pathogenic Pseudomonas species. Int. J. system. Bact. 25, 252-257 (1975).

ELROD, R.P., BRAUN, A.C.: Pseudomonas aeruginosa; its role as a plant pathogen. J. Bact. 44, 633-644 (1942)

EL-SHARKAWY, T.A., HUISINGH, D.: Differentiation among Xanthomonas species by polyacrylamide gel electrophoresis of soluble proteins. J. gen. Microbiol. 68, 155-165 (1971)

ERCOLANI, G.L.: Pseudomonas savastanoi and other bacteria colonizing the surface of the olive leaves in the field. J. gen. Microbiol. 109, 245-257 (1978)

FOX, J.L.: A new bacterial and yeast identification technology. ASM News 53, 469-470 (1987)

FREIFELDER, D.M.: Essentials of molecular biology. Boston, Jones and Bartlett Publishers 1985

FRIEDMAN, S., DE LEY, J.: "Genetic species" concept in Xanthomonas. J. Bact. 89, 95-100 (1965)

FROMME, F.D., MURRAY, T.J.: Angular-leafspot of tobacco, an undescribed bacterial disease. J. agr. Res. 16, 219-228 (1919)

GARRETT, C.M.E.: Bacterial diseases of food plants - an overview, pp. 115-132. In: Bacteria and plants (M.E. RHODES-ROBERTS, F.A. SKINNER, eds.). London, Academic Press 1982

GORDON, A.H.: Electrophoresis of proteins in polyacrylamide and starch gels, pp. 7-207. In: Laboratory techniques in biochemistry and molecular biology (T.S. WORK, E. WORK, eds.), vol. 1. Amsterdam, North Holland 1975

HAMES, B.D.: An introduction to polyacrylamide gel electro= phoresis, pp. 1-91. In: Gel electrophoresis of proteins (B.D. HAMES, D. RICKWOOD, eds.). Oxford, IRL Press 1981

HARRIGAN, W.F., McCANCE, M.E.: Laboratory methods in microbiology. London, Academic Press 1966.

HAYWARD, A.C.: Methods of identification in the genus Xanthomonas, pp. 9-14. In: Identification methods for microbiologists (B.M. GIBBS, F.A. SKINNER, eds.), part A. London, Academic Press 1966

HAYWARD, A.C.: Occurrence of glycoside hydrolases in plant pathogenic and related bacteria. J. appl. Bact. 43, 407-411 (1977)

HILDEBRAND, D.C.: Pectate and pectin gels for differentiation of Pseudomonas spp. and other bacterial plant pathogens. Phytopathology 61, 1430-1436 (1971)

HUGH, R.: Pseudomonas maltophilia sp. nov. nom. rev. Int. J. system. Bact. 30, 7-27 (1981)

HUGH, R., LEIFSON, E.: The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. J. Bact. 66, 24-26 (1953)

JACKMAN, P.J.H.: Classification of Corynebacterium species from axillary skin by numerical analysis of electrophoretic protein patterns. J. med. Microbiol. 15, 485-492 (1982)

JACKMAN, P.J.H.: Bacterial taxonomy based on electrophoretic whole-cell protein patterns, pp. 115-129. In: Chemical methods in bacterial systematics (M. GOODFELLOW, D.E. MINNIKIN, eds.). London, Academic Press 1985

KERSTERS, K., DE LEY, J.: Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. J. gen. Microbiol. 87, 333-342 (1975)

KERSTERS, K., DE LEY, J.: Classification and identification of bacteria by electrophoresis of their proteins, pp. 273-297. In: Microbiological classification and identification (M. GOODFELLOW, R.G. BOARD, eds.). London, Academic Press 1980

KOVACS, N.: Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature 178, 703 (1956)

KRIEG, N.R., JONES, D.: Bacterial classification V. Serology and chemotaxonomy, pp. 979-982. In: Bergey's manual of systematic bacteriology (P.H.A. SNEATH, N.S. MAIR, M.E. SHARPE, J.G. HOLT, eds.), 9th ed., vol. II. London, Williams and Wilkins 1986

LAEMMLI, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685 (1970)

LAZO, G.R., GABRIEL, D.W.: Conservation of plasmid DNA sequences and pathovar identification of strains of Xanthomonas campestris. Phytopathology 77, 448-453 (1987)

LAZO, G.R., ROFFEY, R., GABRIEL, D.W.: Pathovars of Xanthomonas campestris are distinguishable by restriction fragment-length polymorphism. Int. J. system. Bact. 37, 214-221 (1987)

LYSENKO, O.: Pseudomonas - an attempt at a general clas= sification. J. gen. Microbiol. 25, 379-408 (1961)

MAAS, J.L., FINNEY, M.M., CIVEROLO, E.L., SASSER, M.: Association of an unusual strain of Xanthomonas campestris with apple. Phytopathology 75, 438-445 (1985)

MIGULA, W.: Ueber ein neues System der Bakterien. Arb. Bakteriol. Inst. Tech. Hochsch. Karlsruhe 1, 235-238 (1894) (as quoted by Palleroni, 1984)

MOORE, W.E.C., HASH, D.E., HOLDEMAN, L.V., CATO, E.P.: Poly= acrylamide slab gel electrophoresis of soluble proteins for studies of bacterial floras. Appl. Environ. Microbiol. 39, 900-907 (1980)

MULREAN, E.N., SCHROTH, M.M.: Ecology of Xanthomonas campestris pv. juglandis on Persian (English) walnuts. Phytopathology 72, 434-438 (1982)

OWEN, R.J., JACKMAN, P.J.H.: The simmilarities between Pseudomonas paucimobilis and allied bacteria derived from analysis of deoxyribonucleic acids and electrophoretic protein patterns. J. gen. Microbiol. 128, 2945-2954 (1982)

OYAIZU, H., KOMAGATA, K.: Grouping of Pseudomonas species on the basis of cellular fatty acid compositions and the quinone system with special reference to the existence of 3-hydroxy fatty acids. J. gen. appl. Microbiol. 29, 17-40 (1983)

PAINE, S.G.: Studies in bacteriosis. II. A brown blotch disease of cultivated mushrooms. Ann. appl. Biol. 5, 206-219 (1918)

PALLERONI, N.J., BALLARD, R.W., RALSTON, E., DOUDOROFF, M.: Deoxyribonucleic acid homologies among some Pseudomonas species. J. Bact. 110, 1-11 (1972)

PALLERONI, N.J., KUNISAWA, R., CONTOPOULOU, R., DOUDOROFF, M.: Nucleic acid homologies in the genus Pseudomonas. Int. J. system. Bact. 23, 333-339 (1973)

PALLERONI, N.J.: Introduction to the family Pseudomonadacea, pp. 655-665. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (M.P. STARR, H. STOLP, H.G. TRüPER, A. BALOWS, H.G. SCHLEGEL, eds.). Berlin, Springer-Verlag 1981

PALLERONI, N.J.: Family I. Pseudomonadaceae, pp. 141-199. In: Bergey's manual of systematic bacteriology (N.R. KRIEG, J.G. HOLT, eds.), 9th ed., vol. I. Baltimore, Williams and Wilkins 1984

PALMER, B.C., CAMERON, H.R.: Comparison of plant-pathogenic pseudomonads by disc-gel electrophoresis. Phytopathology 61, 984-986 (1971)

ROBBS, C.F.: Una nova doenca bacteriana do mamoeiro (Carica papaya L.). Rev. Soc. Brasil. Agron. 12, 73-76 (1956)

ROBBS, C.F., RIBEIRO, R. DE L.D., KIMURA, O.: Sobre a posicao taxonomica de Pseudomonas mangiferaeindicae Patel et al. 1948, agente causal da "mancha bacteriana" das folhas da mangueira (Mangifera indica L.). Arq. Univ. Fed. Rural Rio de Janeiro 4, 11-14 (1974)

ROHLF, F.J., SOKAL, R.R.: Coefficients of correlation and distance in numerical taxonomy. Univ. Kansas Sci. Bull. 45, 3-27 (1965)

SANDS, D.C., SCHROTH, M.N., HILDEBRAND, D.C.: Taxonomy of phytopathogenic pseudomonads. J. Bact. 101, 9-23 (1970)

SANDS, D.C., SCHROTH, M.N., HILDEBRAND, D.C.: Pseudomonas, pp. 36-44. In: Laboratory guide for identification of plant pathogenic bacteria (M.W. SCHAAD, ed.). Minnesota, Am. Phytopath. Soc. 1980

SCHROTH, M.N., HILDEBRAND, D.C.: Toward a sensible taxonomy of bacterial plant pathogens. Plant Dis. 67, 128 (1983)

SCHROTH, M.N., HILDEBRAND, D.C., STARR, M.P.: Phytopathogenic members of the genus Pseudomonas, pp. 701-718. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (M.P. STARR, H. STOLP, H.G. TRÜPER, A. BARLOWS, H.G. SCHLEGEL, eds.). Berlin, Springer-Verlag 1981

SHIPTON, W.A., FLEISCHMANN, G.: Taxonomic significance of protein patterns of rust species and formae speciales obtained by disc electrophoresis. Can. J. Bot. 47, 1351-1358 (1969)

SIERRA, G.: A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek 23, 15 (1957)

SKERMAN, V.B.D., McGOWAN, V., SNEATH, P.H.A.: Approved lists of bacterial names. Int. J. system. Bact. 30, 225-420 (1980)

SNEATH, P.H.A.: Bacterial classification II. Numerical taxonomy, pp. 969-971. In: Bergey's manual of systematic bacteriology (P.H.A. SNEATH, N.S. MAIR, M.E. SHARPE, J.G. HOLT, eds.), 9th ed., vol. II. London, Williams and Wilkins 1986

SNEATH, P.H.A., SOKAL, R.R.: Numerical taxonomy. The principles and practice of numerical classification. San Francisco, W.H. Freeman and Co. 1973

SOKAL, R.R., MICHENER, C.D.: A statistical method for evaluating systematic relationships. Univ. Kansas Sci. Bull. 38, 1409-1438 (1958)

STARR, M.P.: The genus Xanthomonas, pp. 742-763. In: The prokaryotes: a handbook on habitats, isolation and identi= fication of bacteria (M.P. STARR, H. STOLP, H.G. TRÜPER, A. BALOWS, H.G. SCHLEGEL, eds.). Berlin, Springer-Verlag 1981

STARR, M.P., BURKHOLDER, W.H.: Lipolytic activity of phytopathogenic bacteria determined by means of spirit blue agar and its taxonomic significance. Phytopathology 32, 598-604 (1942)

STARR, M.P., STEPHENS, W.L.: Pigmentation and taxonomy of the
genus Xanthomonas. J. Bact. 87, 293-302 (1964)

STEGEMANN, H., BURGERMEISTER, W., FRANCKSEN, H., KRÖGERRECKLEN-
FORT, E.: Panta-manual on gel electrophoresis and isoelectric
focusing. Braunschweig, Institut für Biochemie, 1987

STEVENS, F.L.: Plant disease fungi. New York, The MacMillan Co.
1925

STEYN, P.L., VILJOEN, N.M., KOTZé, J.M.: The causal organism of
bacterial black spot of mangoes. Phytopathology 64, 1400-1404
(1974)

STOLP, H., STARR, M.P., BAIGENT, N.L.: Problems in speciation of
phytopathogenic pseudomonads and xanthomonads. Ann. Rev.
Phytopathol. 3, 231-264 (1965)

SWINGS, J., DE VOS, P., VAN DEN MOOTER, M., DE LEY, J.: Transfer
of Pseudomonas maltophilia Hugh 1981 to the genus Xanthomonas
as Xanthomonas maltophilia (Hugh 1981) comb. nov. Int. J.
system. Bact. 33, 409-413 (1983)

THORNLEY, M.: The differentiation of Pseudomonas from other
Gram-negative bacteria on the basis of arginine metabolism.
J. appl. Bact. 23, 37 (1960)

VAN DEN MOOTER, M., MARAîTE, H., HEIRESONNE, L., SWINGS, J.,
GILLIS, M., KERSTERS, K., DE LEY, J.: Comparison between
Xanthomonas campestris pv. manihotis (ISPP List 1980), and X.
campestris pv. cassavae (ISPP List 1980) by means of
phenotypic, protein electrophoretic, DNA hybridization and
phytopathological techniques. J. gen. Microbiol. 133, 57-71
(1987)

VAN DEN MOOTER, M., STEENACKERS, M., MAERTENS, C., GOSSELé, F.,
   DE VOS, P., SWINGS, J., KERSTERS, K., DE LEY, J.: Differen=
   tiation between Xanthomonas campestris pv. graminis (ISPP List
   1980), pv. phleipratensis (ISPP List 1980) emend., pv. poae Egli
   and Schmidt 1982 and pv. arrhenatheri Egli and Schmidt 1982, by
   numerical analysis of phenotypic features and protein gel
   electrophoregrams. J. Phytopathol. 118, 135-156 (1987)

VAN ZYL, E., KOTZé, J.M., STEYN, P.L.: Isolation of Xanthomonas
   campestris pv. mangiferaeindicae from gall fly-induced lesions
   on mango leaves. Phytophylactica 20, 89-90 (1988)

VERA CRUZ, C.M., GOSSELé, F., KERSTERS, K., SEGERS, P., VAN DEN
   MOOTER, M., SWINGS, J., DE LEY, J.: Differentiation between
   Xanthomonas campestris pv. oryzae, Xanthomonas campestris pv.
   oryzicola and the bacterial "brown blotch" pathogen on rice by
   numerical analysis of phenotypic features and protein gel
   electrophoregrams. J. gen. Microbiol. 130, 2983-2999 (1984)

VILJOEN, N.M.: Oorsaak, epidemiologie en bestryding van 'n
   bakteriese siekte van Mangifera indica L. M.Sc-thesis, Univ.
   of Pretoria 1972

WILLEMS, A., GILLIS, M., KERSTERS, K., VAN DEN BROECKE, L., DE
   LEY, J.: Transfer of Xanthomonas ampelina Panagopoulos 1969 to
   a new genus, Xylophilus gen. nov., as Xylophilus ampelinus
   (Panagopoulos 1969) comb. nov. Int. J. system. Bact. 37,
   422-430 (1987)

WOESE, C.R., BLANZ, P., HAHN, C.M.: What isn't a pseudomonad:
   the importance of nomenclature in bacterial classification.
   System. appl. Microbiol. 5, 179-195 (1984)

WOLF, F.A., FOSTER, A.C.: Bacterial leaf spot of tobacco.
   Science 46, 361-362 (1917)

YOUNG, J.M., DYE, D.W., WILKIE, J.P.: Genus VII. Pseudomonas
   Migula 1894. In: A proposed nomenclature and classification
   for plant pathogenic bacteria (YOUNG, J.M., DYE, D.W.,
   BRADBURY, J.F., PANAGOPOULOS, C.G., ROBBS, C.F.). N.Z. J.
   agric. Res. 21, 153-177 (1978)

ZUPAN, J.: Clustering of large data sets (D. BAWDEN ed.).
   Chichester, Research Studies Press 1982

CHAPTER III

TAXONOMY OF THE PHYTOPATHOGENIC PSEUDOMONAS SPECIES BELONGING

TO THE ACIDOVORANS rRNA COMPLEX

SUMMARY

The taxonomic relationships of representatives of the majority of the phytopathogenic Pseudomonas species belonging to the acidovorans rRNA complex were determined by numerical analysis of polyacrylamide gel electrophoregrams of soluble proteins. Two well defined clusters were formed, with a very low correlation between them. "[P.] setariae" was found not to be synonymous with [P.] avenae, but probably represents a different species closely related to [P.] rubrilineans. [P.] avenae (NCPPB 2402) is probably misnamed.

INTRODUCTION

The elucidation of relationships among bacteria at the generic and suprageneric levels is one of the main problems to be solved in modern bacterial taxonomy (DE VOS and DE LEY, 1983). For this reason much attention has been devoted recently to deoxyribonucleic acid – ribosomal ribonucleic acid (DNA – rRNA) hybridization and rRNA sequence studies. However, the elucidation of relationships at the subgeneric level is equally important for the classification and identification of strains. Most of the recent taxonomic studies of Pseudomonas species have been concerned with nucleic acid analysis (DE VOS and DE LEY, 1983; DE VOS et al., 1985; DE VOS et al., 1989). Very few studies have been carried out to revise and improve the phenotypic definitions of species and to determine their generic positions and subgeneric relationships (GAVINI et al., 1989).

Presently, Section III of Pseudomonas (the [Pseudomonas] acidovorans rRNA branch) in Bergey's Manual of Systematic Bacteriology (PALLERONI, 1984) contains no phytopathogens. Recent DNA-rRNA hybridization studies, however, have assigned the following phytopathogens to this rRNA branch (since all of these Pseudomonas spp. are considered to be generically misnamed, this is indicated by brackets): [Pseudomonas] avenae (syn. "[P.] alboprecipitans"), [Pseudomonas] cattleyae, [Pseudomonas]

pseudoalcaligenes subsp. citrulli, [Pseudomonas] pseudoalcaligenes subsp. konjaci, [Pseudomonas] rubrilineans and "[Pseudomonas] setariae" (BYNG et al., 1980; DE VOS et al., 1985; GOOR et al., 1986; WILLEMS et al., 1987). As the results of DNA-rRNA hybridization studies do not generally allow species separation within a rRNA branch (DE VOS et al., 1985), knowledge of the natural relationships between these species is incomplete.

In a previous study of phytopathogenic Pseudomonas and Xanthomonas species (VAN ZYL and STEYN, 1990), it was shown that numerical analysis of electrophoretic patterns of the total soluble proteins of the bacterial cell is a reliable tool for establishing taxonomic relationships among phytopathogenic pseudomonads and xanthomonads from the level of pathovar to genus. The results of this study correlated well with those obtained from classical phenotypic features, DNA homology and DNA-rRNA hybridization studies.

This study deals with the taxonomic relationships of the majority of the phytopathogenic species of the acidovorans rRNA branch as determined by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) of soluble proteins.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The strains used are listed in Table 1. The bacteria were grown at 28°C on GYCA medium (VERA CRUZ et al., 1984) and maintained on GYCA slants at room temperature. For electrophoretic studies cultures were incubated with shaking at 28°C for 72 h in 10 ml Standard 1 nutrient broth (Merck) supplemented with 0,15% K_2HPO_4 and 0,15% $Mg_2SO_4 .7H_2O$.

Legend to Table 1.   Strains used.

(a)   All misnamed taxa are indicated by brackets.

(b)   NCPPB: National Collection of Plant Pathogenic Bacteria,
      Harpenden, England.

(c)   [Pseudomonas] acidovorans was recently transferred to the
      genus Comamonas (TAMAOKA et al., 1987)

(d)   Name not retained on the Approved Lists of Bacterial Names
      (SKERMAN et al., 1980; MOORE et al., 1985) or Validation
      Lists 17 to 29 (International Journal of Systematic
      Bacteriology, 1985-1989)

Table 1.   Strains used.

Species[a]	Strain[b]	Source, place and year of isolation		
Comamonas acidovorans[c]	NCPPB 1967	Pharyngeal swab,		1966
[Pseudomonas] avenae	NCPPB 2398	Bromus catharticus,	Japan,	1966
[Pseudomonas] avenae	NCPPB 2400	Euchlaena mexicana,	Japan,	1967
[Pseudomonas] avenae	NCPPB 2402	Bromus inermis,	Japan,	1967
[Pseudomonas] avenae	NCPPB 2403	Agropyron trichophorum,	Japan,	1966
[Pseudomonas] avenae	NCPPB 3354	Oryza sativa seed,	India,	1984
[Pseudomonas] avenae	NCPPB 3355	Oryza sativa seed,	Brazil,	1984
[Pseudomonas] avenae	NCPPB 3356	Oryza sativa seed,	Nepal,	1984
[Pseudomonas] avenae	NCPPB 3358	Oryza sativa seed,	Turkey,	1984
[Pseudomonas] pseudo-alcaligenes subsp. citrulli	NCPPB 3244	Citrullus lanatus,	USA,	1978
[Pseudomonas] rubrilineans	NCPPB 920	Saccharum officinarum,	Reunion,	1960
"[Pseudomonas] setariae"[d]	NCPPB 1392	Oryza sativa,	Japan,	1955

Preparation of whole cell extracts for electrophoresis

Whole cell protein extracts were prepared as described by VAN ZYL and STEYN (1990), with the following modification: 0,01 g (wet weight) of harvested cells from each strain was resuspended in 1 ml sample buffer (LAEMMLI, 1970) in an Eppendorf centrifuge tube and sonicated for 30 s at a 40 watt output with a Cole-Parmer ultrasonic processor (series 4710) fitted with a 3 mm microtip.

Electrophoresis, densitometry and numerical analysis of protein patterns

One-dimensional vertical electrophoresis, using the discontinuous buffer system of LAEMMLI (1970), was performed as previously described (VAN ZYL and STEYN, 1990). Destained gels were scanned with a Hoefer GS 300 scanning densitometer connected to a Lloyd Graphic 1000 Y/t strip chart recorder. The wavelength used was 580 nm and the chart speed 5 mm/s. The densitometric scans were normalized by the method of KERSTERS and DE LEY (1975). Computer-assisted numerical analysis of normalized densitometric tracings was done by the procedure of VAN ZYL and STEYN (1990).

RESULTS AND DISCUSSION

The dendrogram obtained after numerical analysis of the data is shown in Fig 1. Two well defined clusters were formed. Cluster I contained Comamonas acidovorans, [P.] pseudoalcaligenes subsp. citrulli, [P.] rubrilineans and "[P.] setariae"; cluster II all the [P.] avenae strains.

Although located in cluster I, C. acidovorans, the only non-phytopathogenic species included in this study, was only distantly related to the phytopathogens of this cluster (r = 0,40).

Fig. 1.   Dendrogram of the correlation coefficient, r, grouped
          by the unweighted average linkage cluster analysis,
          showing the taxonomic relationships between several
          phytopathogenic species of the acidovorans rRNA
          complex.

Fig. 1.
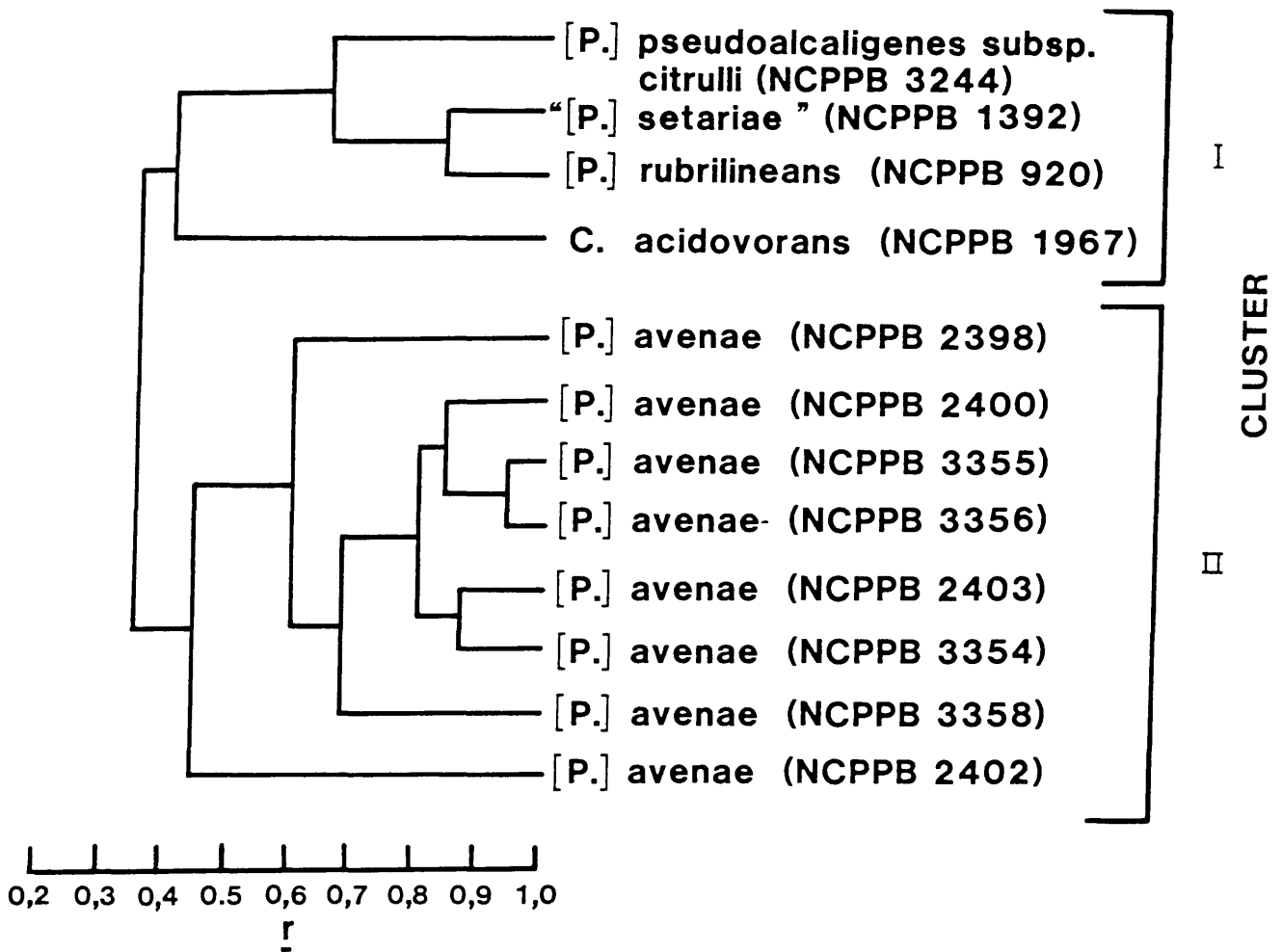
These results are in partial agreement with those of WILLEMS et al. (1987). These authors proposed an acidovorans rRNA subbranch containing C. acidovorans, with [P.] pseudoalcaligenes subsp. citrulli, [P.] rubrilineans and "[P.] setariae" located on the $T_{m(e)}$ dendrogram at the branching point of the acidovorans subbranch. [P.] avenae, on the other hand, was located close to the [Alcaligenes] paradoxus rRNA subbranch, thus substantially removed from the other phytopathogens (WILLEMS et al., 1987). On the basis of cellular fatty acid composition and the quinone system, OYAIZU and KOMAGATA (1983) also placed C. acidovorans and [P.] avenae in separate groups. We therefore cannot agree with WILLEMS et al. (1989) that [P.] avenae is closely related to [P.] rubrilineans and "[P.] setariae". The [P.] rubrilineans and "[P.] setariae" strains used by us were the same as those used by WILLEMS et al. (1989) but the [P.] avenae strains were different.

The low correlation between the two clusters in Fig 1 (r = 0,35) supports the postulation that each subbranch in the acidovorans rRNA complex deserves generic rank and that many species of this group are therefore generically misnamed (WILLEMS et al., 1987). The low correlation between species within a cluster also indicates that the organisms of the acidovorans rRNA complex show great diversity (WILLEMS et al., 1987; DE VOS et al., 1989).

The taxonomic status of "[Pseudomonas] setariae" is not clear. The species name was not retained in the Approved Lists of Bacterial Names (SKERMAN et al., 1980; MOORE et al., 1985), nor is it on Validation Lists 17 to 29 (International Journal of Systematic Bacteriology, 1985-1989). In the catalogue of the National Collection of Plant Pathogenic Bacteria (1988) "[P.] setariae" (NCPPB 1392) is listed as a synonym of [P.] avenae. However, it is noted that this culture has not been checked by the NCPPB nor by a recognised authority and that its authenticity is doubtful. According to our results "[P.] setariae" (NCPPB 1392) is not a synonym of [P.] avenae, but a different and separate species. I am therefore tempted to suggest that the

name "[P.] setariae" should be revived as cultures are available and the name is in general use (DE VOS et al., 1985; WILLEMS et al., 1987; WILLEMS et al., 1989). However, the high relatedness found between "[P.] setariae" and [P.] rubrilineans (r = 0,83) on the basis of their protein electrophoretic patterns, suggests a substantial degree of genome similarity between these two taxa. This should be investigated further by other techniques before a nomenclatural change is proposed.

The placement of [P.] pseudoalcaligenes subsp. citrulli in cluster I with [P.] rubrilineans and "[P.] setariae" is in agreement with the results of SCHAAD et al. (1978). These workers also found this watermelon bacterium to fit most closely into group III of the plant pathogenic pseudomonads as proposed by SANDS et al. (1970); a group that included [P.] rubrilineans and "[P.] setariae".

Except for [P.] avenae (NCPPB 2402) all the [P.] avenae strains clustered above r = 0,60. Because of the low correlation of strain NCPPB 2402 with the other [P.] avenae strains (r = 0,44) and because its authenticity is not known to have been verified (National Collection of Plant Pathogenic Bacteria, 1988), we question the identity of this strain and suggest that it should be re-examined.

In the [P.] avenae cluster (cluster II) no correlation was found between electrophoretic clustering and host plant or geographic origin.

As in the past, the results agreed well with those of other techniques, again accentuating the reliability and applicability of PAGE as a taxonomic tool in everyday phytobacterial practices.

The combination of these results with the results of other techniques, such as DNA-rRNA hybridization data, should contribute to a better understanding of the relationships within the acidovorans rRNA complex.

REFERENCES

BYNG, G.S., WHITAKER, R.J., GHERNA, R.L., JENSEN, R.A.: Variable enzymological patterning in tyrosine biosynthesis as a means of determining natural relatedness among the Pseudomonadaceae. J. Bacteriol. 144, 247-257 (1980)

DE VOS, P., DE LEY, J.: Intra- and intergeneric similarities of Pseudomonas and Xanthomonas ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 33, 487-509 (1983)

DE VOS, P., GOOR, M., GILLIS, M., DE LEY, J.: Ribosomal ribonucleic acid cistron similarities of phytopathogenic Pseudomonas species. Int. J. system. Bact. 35, 169-184 (1985)

DE VOS, P., VAN LANDSCHOOT, A., SEGERS, P., TYTGAT, R., GILLIS, M., BAUWENS, M., ROSSAU, R., GOOR, M., POT, B., KERSTERS, K., LIZZARAGA, P., DE LEY, J.: Genotypic relationships and taxonomic localization of unclassified Pseudomonas and Pseudomonas-like strains by deoxyribonucleic acid: ribosomal ribonucleic acid hybridizations. Int. J. system. Bact. 39, 35-49 (1989)

GAVINI, F., HOLMES, B., IZARD, D., BEJI, A., BERNIGUAD, A., JAKUBCZAK, E.: Numerical taxonomy of Pseudomonas alcaligenes, P. pseudoalcaligenes, P. mendocina, P. stutzeri, and related bacteria. Int. J. system. Bact. 39, 135-144 (1989)

GOOR, M., KERSTERS, K., GILLIS, M., THIELEMANS, S., DE LEY, J.: Taxonomy of the phytopathogenic Pseudomonas species belonging to the P. acidovorans rRNA branch, p. 37. In: Proceedings of the XIV Int. Congress of Microbiol., England. 1986.

INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY: Validation of the publication of new names and new combinations previously effectively published outside the IJSB. Lists no. 17-29. Int. J. system. Bact. 35-39 (1985-1989)

KERSTERS, K., DE LEY, J.: Identification and grouping of bacteria by numerical analysis of their electrophorectic protein patterns. J. gen. Microbiol. <u>87</u>, 333-342 (1975)

LAEMMLI, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature <u>227</u>, 680-685 (1970)

MOORE, W.E.C., CATO, E.P., MOORE, L.V.H.: Index of bacterial and yeast nomenclatural changes published in the International Journal of Systematic Bacteriology since the 1980 Approved Lists of Bacterial Names (1 January 1980 - 1 January 1985). Int. J. system. Bact. <u>35</u>, 382-407 (1985)

NATIONAL COLLECTION OF PLANT PATHOGENIC BACTERIA: <u>Pseudomonas</u> Migula 1894, pp. 27-36. In: Catalogue of strains - National Collection of Plant Pathogenic Bacteria, 2nd ed. England, Adas laboratory services 1988

OYAIZU, H., KOMAGATA, K.: Grouping of <u>Pseudomonas</u> species on the basis of cellular fatty acid compositions and the quinone system with special reference to the existence of 3-hydroxy fatty acids. J. gen. appl. Microbiol. <u>29</u>, 17-40 (1983)

PALLERONI, N.J.: Family I. <u>Pseudomonadaceae</u>, pp. 141-199. In: Bergey's manual of systematic bacteriology (N.R. Krieg, J.G. Holt, eds.), 9th ed., Vol. I. Baltimore, Williams and Wilkins 1984

SANDS, D.C., SCHROTH, M.N., HILDEBRAND, D.C.: Taxonomy of phytopathogenic pseudomonads. J. Bact. <u>101</u>, 9-23 (1970)

SCHAAD, N.W., SOWELL, G., GOTH, R.W., COLWELL, R.R., WEBB, R.E.: <u>Pseudomonas</u> <u>pseudoalcaligenes</u> subsp. <u>citrulli</u> subsp. nov. Int. J. system. Bact. <u>28</u>, 117-125 (1978)

SKERMAN, V.B.D., MCGOWAN, V., SNEATH, P.H.A.: Approved lists of
bacterial names. Int. J. system. Bact. 30, 225-420 (1980)

TAMAOKA, J., HA, D-M, KOMAGATA, K.: Reclassification of
Pseudomonas acidovorans den Dooren de Jong 1926 and Pseudomonas
testosteroni Marcus and Talalay 1956 as Comamonas acidovorans
comb. nov. and Comamonas testosteroni comb. nov., with an
emended description of the genus Comamonas. Int. J. system.
Bact. 37, 52-59 (1987)

VAN ZYL, E., STEYN, P.L.: Differentiation of phytopathogenic
Pseudomonas and Xanthomonas species and pathovars by numerical
taxonomy and protein gel electrophoregrams. System. Appl.
Microbiol. 13, 60-71 (1990)

VERA CRUZ, C.M., GOSSELé, F., KERSTERS, K., SEGERS, P., VAN DEN
MOOTER, M., SWINGS, J., DE LEY, J.: Differentiation between
Xanthomonas campestris pv. oryzae, Xanthomonas campestris pv.
oryzicola and the bacterial "brown blotch" pathogen on rice by
numerical analysis of phenotypic features and protein gel
electrophoregrams. J. gen. Microbiol. 130, 2983-2999 (1984)

WILLEMS, A., GILLIS, M., KERSTERS, K., VAN DEN BROECKE, L., DE
LEY, J.: Transfer of Xanthomonas ampelina Panagopoulos 1969 to
a new genus, Xylophilus gen. nov., as Xylophilus ampelinus
(Panagopoulos, 1969) comb. nov. Int. J. system. Bact. 37,
422-430 (1987)

WILLEMS, A., BUSSE, J., GOOR, M., POT, B., FALSEN, E., JANTSEN,
E., HOSTE, B., GILLIS, M., KERSTERS, K., AULING, G., DE LEY,
J.: Hydrogenophaga, a new genus of hydrogen-oxidizing bacteria
that includes Hydrogenophage flava comb. nov. (formerly
Pseudomonas flava), Hydrogenophaga palleronii, (formerly
Pseudomonas palleroni), Hydrogenophaga pseudoflava (formerly
Pseudomonas pseudoflava and "Pseudomonas carboxydoflava"), and
Hydrogenophaga taeniospiralis (formerly Pseudomonas
taeniospiralis). Int. J. system. Bact. 39, 319-333 (1989)

CHAPTER IV

REINTERPRETATION OF THE TAXONOMIC POSITION OF XANTHOMONAS MALTOPHILIA AND TAXONOMIC CRITERIA IN THIS GENUS

SUMMARY

The inclusion of "Pseudomonas" maltophilia in the genus Xanthomonas as Xanthomonas maltophilia is questioned in view of the significant differences between these two taxons. This reclassification is not acceptable if practical means of differentiation in this genus is considered. The proposed alteration of the genus description of Xanthomonas is also questionable because of the implications for everyday phytobacteriology. In view of the natural similarities, as well as the profound differences between X. maltophilia and Xanthomonas, it is proposed that a new genus is created for X. maltophilia, which could be placed together with Xanthomonas in a separate natural group.

INTRODUCTION

The taxonomic position of Xanthomonas maltophilia has been the subject of much debate (PALLERONI et al., 1973; IKEMOTO et al., 1980, PALLERONI, 1981; STARR, 1981; SWINGS et al., 1983; DE VOS and DE LEY, 1983; BRADBURY, 1984; PALLERONI, 1984; JOHNSON and PALLERONI, 1989). Initially isolated from pleural fluid in 1943 and named "Bacterium bookerii", this ubiquitous bacterium was reclassified as Pseudomonas maltophilia by HUGH and RYSCHENKOW (1961). As "P". maltophilia and species of Xanthomonas share a substantial level of rRNA homology (PALLERONI et al., 1973), it appeared appropriate to assign this species to the genus Xanthomonas (PALLERONI, 1984). As a number of arguments in the literature also support this view, SWINGS et al. (1983) proposed the transfer of P. maltophilia Hugh 1981 to the genus Xanthomonas as Xanthomonas maltophilia (Hugh 1981) comb. nov.

In view of the significant differences between these two taxons, this reclassification remains questionable (PALLERONI, 1986), partly because of the redefinition of the genus Xanthomonas that will be required to accommodate X. maltophilia (BRADBURY, 1984; PALLERONI, 1984).

As own results (VAN ZYL and STEYN, 1990) also argue against inclusion of X. maltophilia in the genus Xanthomonas, the purpose of this study is to question some of the assumptions made by SWINGS et al. (1983), to summarise the latest information on this subject, and to request an opinion.

The following arguments proceed from the reclassification proposed by SWINGS et al. (1983) and should be considered together with their corresponding discussions:

DNA-rRNA HYBRIDIZATIONS: The taxonomic value and reliability of this technique for the classification of bacteria on generic and suprageneric levels have been demonstrated by several workers in this field (DE SMEDT and DE LEY, 1977; DE LEY et al., 1978; DE SMEDT et al., 1980; GILLIS and DE LEY, 1980; DE VOS and DE LEY, 1983; DE VOS et al., 1985). However, from the reclas= sification proposed by SWINGS et al. (1983), the following are not clear: although SWINGS et al. (1983) refer to an extensive DNA-rRNA hybridization study of the genus Pseudomonas by De Vos and De Ley, different $T_{m(e)}$ ranges are reported by these authors for exactly the same Xanthomonas strains. SWINGS et al. (1983) reported a range of 78,8 to 81,5°C, while DE VOS and DE LEY (1983) found it to be 80,0 to 81,5°C for the same 27 Xanthomonas strains under identical conditions and using the same reference rRNA. In the case of SWINGS et al. (1983) the range determined for X. maltophilia (76,5 to 78,0°C) (DE VOS and DE LEY, 1983; SWINGS et al., 1983) will therefore almost overlap with that of Xanthomonas, whereas DE VOS and DE LEY (1983) found X. maltophilia to be removed from Xanthomonas at a $T_{m(e)}$ of 3°C under these conditions. This places the X. maltophilia cluster close to the genus Xanthomonas, but still out of range. Also

when using reference rRNA from three <u>Pseudomonas</u> type strains, <u>X. maltophilia</u> was located close to <u>Xanthomonas</u>, but never included in the same similarity cluster (DE VOS and DE LEY, 1983).

As the amount of hybrid formation depends not only on the actual sequence homology, but also on the size of the genome, its state of replication and the number of rRNA cistrons per genome, rRNA similarity should be expressed by two parameters, percent rRNA binding and melting point [$T_{m(e)}$] of DNA-rRNA hybrids (KANDLER and SCHLEIFER, 1980). SWINGS et al. (1983) did not mention the % rRNA binding in this case. Although the percentage of rRNA binding might not be a direct measure of rRNA cistron homology, it is a useful parameter to separate taxa with the same $T_{m(e)}$ value (DE VOS et al., 1985), in this case removing <u>X. maltophilia</u> even further from the extremely tight <u>Xanthomonas</u> cluster when plotted on a similarity map (DE VOS and DE LEY, 1983).

As DNA-rRNA hybridizations were the first indicators of a possible transfer of "<u>P</u>". <u>maltophilia</u> to the genus <u>Xanthomonas</u> (PALLERONI et al., 1973), it is of interest to note the opinion of WOESE et al. (1984) on the DNA-rRNA approach. According to them the DNA-rRNA approach offers no advantage over DNA-DNA hybridization (which already revealed low levels of DNA similarity among <u>Pseudomonas</u> species (JOHNSON and PALLERONI, 1989)) when it comes to groupings at the generic level, and has the disadvantage that it samples only one or two genes. In their opinion the DNA-rRNA approach should soon become obsolete as it offers neither the precision nor the extent of data obtainable with sequencing approaches for example.

DNA-DNA HOMOLOGY: At present DNA-DNA homology is widely accepted as an expression of genetic relatedness (IKEMOTO et al., 1980). Membrane competition experiments, however, revealed very low levels of DNA similarity among <u>Pseudomonas</u> species of the five different rRNA subgroups (JOHNSON and PALLERONI, 1989).

Interspecies similarity values are in the 5 to 20% range, with the average DNA similarity values between species belonging to different rRNA similarity clusters even lower (not more than 1 to 2%) (JOHNSON and PALLERONI, 1989). This presents a practical problem for the classification of species belonging to this group of bacteria, because, although it is not always clear at what point on the homology scale the lines for interspecies and intragenus relatedness should be drawn (GARDNER and KADO, 1972), it is generally accepted that the genosubspecies would be those strains that have a minimum of 60 to 70% DNA homology (JOHNSON, 1973; VAUTERIN et al., 1990). Unfortunately, there is currently no satisfactory phylogenetic definition of a genus (WAYNE et al., 1987). For X. maltophilia the percentage similarity with labeled DNA from X. campestris strains ranges from 22 to 37% (IKEMOTO et al., 1980) or 1 to 16% with the S1 nuclease methodology (JOHNSON and PALLERONI, 1989). Such low values are at the border of or below the sensitivity of this method (SWINGS et al., 1983) and very difficult to interpret.

The low levels of DNA similarity between Pseudomonas species has far reaching implications for taxonomy and phylogenetic relationships in this group, when it is considered that the DNA-DNA approach actually represents (an average of) the entire genome, and not merely a few genes therein (WOESE et al., 1984). For classification purposes it is now also considered necessary to calibrate techniques for the differentiation of strains against DNA-DNA hybridization, the conventional method used for the delineation of species (VAUTERIN et al., 1990).

DNA-DNA hybridization data divided rRNA group V strains into two subgroups: a X. maltophilia subgroup and a X. campestris subgroup, with substantial heterogeneity among the strains in each subgroup (JOHNSON and PALLERONI, 1989). IKEMOTO et al. (1980) also observed heterogeneity among X. maltophilia strains and delineated five clusters on the basis of DNA-DNA homology. There was also a difference between methionine-requiring and nonrequiring strains of X. maltophilia with respect to DNA-DNA homology (IKEMOTO et al., 1980).

GUANINE-PLUS-CYTOSINE CONTENTS: The G + C range determined for X. maltophilia (63-67,5 mol%) fit well into the Xanthomonas range (63-71 mol%) (BRADBURY, 1984), and although it will also fit into the Pseudomonas range (58-70 mol%) (PALLERONI, 1984), these values are not inconsistent with inclusion of X. maltophilia in Xanthomonas. It is, however, also important to bear in mind that although these mol% G + C values are closely similar for related organisms, the reverse is not true: two organisms with similar mol% G + C are not necessarily related (DE LEY, 1968).

COMPARATIVE ENZYMOLOGY: According to SWINGS et al. (1983), "both P. maltophilia and Xanthomonas are unusual in that they lack NADP-linked dehydrogenases and possess NAD-specific prephenate and arogenate dehydrogenases for tyrosine biosynthesis". Referring to the original article on this topic (BYNG et al., 1980) it is learnt that rRNA homology groups I, IV and V all lack activity for arogenate/NADP dehydrogenase, while groups I and V also lack prephenate/NADP dehydrogenase. As both groups I and V furthermore possess NAD-specific prephenate and arogenate dehydrogenases for tyrosine biosynthesis (BYNG et al., 1980), X. maltophilia and Xanthomonas are not so unique in this regard. Groups I and V are separated by comparison of the relative allosteric sensitivities of NAD-dependent dehydrogenases (BYNG et al., 1980). Unfortunately results for X. maltophilia were not included in the comparison of the sensitivities of the NAD-dependent dehydrogenases to tyrosine inhibition (Table 5 - BYNG et al., 1980) and was only one Pseudomonas species (P. gardneri) grouped with all the Xanthomonas species studied (BYNG et al., 1980).

The control pattern for the enzyme 3-deoxy-D-arabinoheptulo-sonate-7-phosphate (DAHP) synthetase is a useful indicator of taxonomic relationship at about the level of genus (WHITAKER et al., 1981). The occurrence of this unique control mechanism in X. maltophilia as well as species of Xanthomonas indeed supports a close taxonomic relationship between these two taxa (WHITAKER

et al., 1981; SWINGS et al., 1983), but it has to be remembered that the comparative allostery of DAHP synthetase alone does not unambiguously establish group placement (WHITAKER et al., 1981).

UBIQUINONES: SWINGS et al. (1983) stated that both X. maltophilia and Xanthomonas contain ubiquinones with eight isoprene units (Q-8), whereas all other Pseudomonas strains tested generally contain ubiquinones with nine isoprene units (Q-9) as major components. In my opinion the above statement does not reflect the true situation correctly. OYAIZU and KOMAGATA (1983) examined the quinone system of 75 Pseudomonas strains and divided them into nine groups according to this characteristic, as well as fatty acid composition. Of the nine groups determined only group I (corresponding to rRNA homology group I) species contain a Q-9 system, while five groups of named pseudomonads contain Q-8 quinone systems. These include a P. solanacearum group (rRNA group II), a "P". acidovorans group (rRNA group III), X. maltophilia (rRNA group V), P. avenae and P. palleronii (rRNA group III). The three other groups contain Q-10 systems (OYAIZU and KOMAGATA, 1983).

Based on ubiquinones, the X. maltophilia-Xanthomonas group is not as unique as implied by SWINGS et al. (1983).

CELLULAR FATTY ACID COMPOSITION: Quantitative differences were found between the fatty acids of X. maltophilia strains that did not require methionine and methionine-requiring strains of this species, although they share almost the same kinds of fatty acids (IKEMOTO et al., 1980). Based on cellular fatty acid compositions a similarity value of 82% was calculated for the two groups (IKEMOTO et al., 1980). From the data by IKEMOTO et al. (1980) it is interesting to note that X. maltophilia strains which do not require methionine also share relatively high mean percentages of i-$C_{16:0}$ and $C_{17:0}$ fatty acids with the two Xanthomonas strains (X. oryzae excluded), values considerably higher than the percentages determined for methionine-requiring strains of X. maltophilia. X. oryzae has a fatty acid

composition quite different from that of X. maltophilia (IKEMOTO et al., 1980).

PHAGE TYPING: Phage typing studies provided evidence for a relationship between X. maltophilia and Xanthomonas (SWINGS et al., 1983). This technique was, however, not very successful as a taxonomic tool in Xanthomonas, mainly because of lack of specificity (GARRETT, 1982; BRADBURY, 1984).

In addition temperate phages have been found in X. maltophilia (PALLERONI, 1984), while nothing to this nature has been reported for Xanthomonas.

GROWTH AND PIGMENTS: The colonies of Xanthomonas species are distinctively yellow in colour, and this pigmentation is very important in their presumptive identification (STARR, 1981). It is suggested that these xanthomonadins might well serve as adequate chemotaxonomic markers for the genus Xanthomonas (STARR, 1981). X. maltophilia cultures do not show the yellow mucoid growth typical of Xanthomonas, and its pigments are not xanthomonadins or closely related to the xanthomonadins already explored (STARR, 1981; SWINGS et al., 1983; PALLERONI, 1984).

Nonpigmented Xanthomonas strains do exist, but evidence in the literature indicate that pigmented forms exist for all the nonpigmented Xanthomonas species which occur naturally (SABET, 1959; ROBBS et al., 1972; STARR and STEPHENS, 1964; ROBBS et al., 1974).

SWINGS et al. (1983) report that some X. maltophilia strains characteristically form brownish grey water-soluble pigments, also observed in six pathovars of X. campestris. GILARDI (1971) is of the opinion that the brown coloration of the culture medium associated with X. maltophilia and previously described as a pigment, is probably due to a secondary chemical reaction among extracellular products which react to form the brown colour.

When grown on media containing usable carbohydrates, most Xanthomonas strains produce characteristic extracellular polysaccarides or xanthan gums, resulting in very mucoid colonies (SWINGS et al., 1983; BRADBURY, 1984). The growth of X. maltophilia is shiny, but not mucoid (SWINGS et al., 1983). This presents a practical problem, as slime formation has some value in screening Xanthomonas cultures (DYE, 1962).

NICHES: All species currently included in the genus Xanthomonas are highly specialised plant pathogens (BRADBURY, 1984), while at present nothing is known about the phytopathogenic capacity of X. maltophilia strains (SWINGS et al., 1983). Most X. maltophilia strains are isolated from clinical specimens - X. maltophilia is the second most frequently isolated Pseudomonas species in the clinical laboratory (PALLERONI, 1984). Strains of this species appear to be opportunistic human pathogens (PALLERONI, 1984). There is presently no evidence of any Xanthomonas species associated with human infections.

X. maltophilia strains are also found in frozen food, milk and water, while X. maltophilia predominates over other pseudomonads in the rhizospheres of several cultivated plants, probably due to the excretion of S-containing amino acids by the roots (PALLERONI, 1984). For Xanthomonas species, however, the minimum growth temperature is above 5°C (BRADBURY, 1984). Xanthomonads have occasionally been detected in runn-off water and ditches around fields of infected plants, but results indicate that survival in this situation would be short (BRADBURY, 1984). For Xanthomonas species, survival in soil saprophytically is unusual (BRADBURY, 1984). Two pathovars of X. campestris are, however, known to spend the interseasonal time epiphytically (BRADBURY, 1984).

OTHER MORPHOLOGICAL, PHYSIOLOGICAL, AND BIOCHEMICAL CHARACTERISTICS: From the literature it is known that X. maltophilia and Xanthomonas share a number of morphological,

physiological and biochemical characteristics (SWINGS et al., 1983). SWINGS et al. (1983) calculated a simple matching similarity coefficient (S_{SM}) of approximately 75% between these two taxa from the available phenotypic data.

In spite of a high similarity value (S_{SM}) of 79% calculated for X. maltophilia in a phenotypic study using 53 standard phenotypic tests, X. maltophilia was less related to the Xanthomonas group than the majority of Pseudomonas strains studied (VAN ZYL and STEYN, 1990). This was confirmed by the results of the numerical analysis of electrophoretic protein patterns of the same strains, where a correlation of r = 0,65 was determined between X. maltophilia and the bigger Pseudomonas/ Xanthomonas cluster (VAN ZYL and STEYN, 1990).

The number of polar flagella is an important taxonomic character (PALLERONI, 1984). The existence of a single polar flagellum is an important characteristic of Xanthomonas. Very rarely cells occur with two polar flagella (BRADBURY, 1984). On the other hand X. maltophilia is multitrichously flagellated (SWINGS et al., 1983; PALLERONI, 1984).

At present no pili or fimbriae have been reported for Xanthomonas (BRADBURY, 1984), while polarly inserted pili or fimbriae were reported for X. maltophilia (PALLERONI, 1984).

According to the present generic definition of Xanthomonas, no nitrate reducing occurs (BRADBURY, 1984). X. maltophilia strains reduce nitrates to nitrites (KOMAGATA et al., 1974; SWINGS et al., 1983).

STARR (1946) and DYE (1962) reported that asparagine is inadequate as a sole source of carbon and nitrogen for xanthomonads. BRADBURY (1984) included this property in the definition of the genus Xanthomonas and this is presently used as a diagnostic test for Xanthomonas, since yellow Enterobacteriaceae and many Pseudomonas species will grow with

asparagine as sole source of both carbon and nitrogen (BRADBURY, 1984). X. maltophilia (NCPPB 1974) also grows vigorously under these conditions (VAN ZYL and STEYN, 1990).

Some of the characteristics shared by X. maltophilia and Xanthomonas according to SWINGS et al. (1983) are acid production from (amongst others) lactose, fructose and maltose, as well as hydrolysis of aesculin. According to BRADBURY (1984) there is actually a variation in the reactions: for acid production from lactose and maltose three Xanthomonas species gave negative reactions, while 11-89% of the X. campestris strains were positive; for acid production from fructose, two Xanthomonas species gave negative reactions, while three species were positive. Hydrolysis of aesculin also gave a varied reaction, with three species positive and one Xanthomonas species negative (BRADBURY, 1984).

Other differences between X. maltophilia and Xanthomonas include the production of lysine decarboxylase, starch hydrolysis and resistance to antibiotics, dyes and metals (SWINGS et al., 1983).

From the above it is clear that profound differences exist between these two taxons; differences which are not without substantial practical implications for the already complex taxonomic structure and identification system in Xanthomonas.

ADDITIONAL REMARKS: Since the publication of the proposal for the transfer of "P". maltophilia to the genus Xanthomonas (SWINGS et al., 1983), the following appeared in favour of or as an argument against this inclusion:

Oligonucleotide cataloging of 16S rRNA : S_{AB} analysis of oligonucleotide cataloging showed X. maltophilia and "its close relatives (i.e. the xanthomonads)" to cluster peripherally with the fluorescent pseudomonas group (P. fluorescens, P. aeruginosa, P. syringae etc.) in particular (WOESE et al., 1984). Catalogs

of the X. maltophilia group, however, also have a remarkable number of oligonucleotides in common with group II species ("P". acidovorans, P. testosteroni, P. cepacia etc.) (WOESE et al., 1984).

Respiratory chain: Difference spectra at liquid air temperature showed a slight shoulder for Xanthomonas at 549 nm, suggesting a very small content of cytochrome c. The difference spectra for P. syringae and X. maltophilia are very similar, but do not show any noticeable peak or shoulder at or near 549 nm (BRADBURY, 1984).

Growth factor requirement: Many Xanthomonas strains require growth factors, usually amino acids such as methionine and/or glutamic acid. Nicotinic acid is occasionally required (BRADBURY, 1984). X. maltophilia has a requirement for methionine, although strains of X. maltophilia which do not require methionine have been described (IKEMOTO et al., 1980). These methionine-requiring and -nonrequiring strains of X. maltophilia differ from each other with respect to phenotypic characteristics, cellular fatty acid composition, DNA base composition and DNA-DNA hybridization, and are separable into two clusters on the basis of methionine requirement and some other properties (IKEMOTO et al., 1980). It is also noteworthy that the requirement for methionine depended upon the carbon compounds employed (IKEMOTO et al., 1980). Although it is likely that the genus Pseudomonas will be reserved in the future for species not requiring growth factors, there are also a few Pseudomonas species that require the addition of organic growth factors. P. diminuta for example also needs methionine, while growth factor-requiring strains of P. caryophylli and P. syringae are occasionally encountered in nature (PALLERONI, 1984).

Metabolism of aromatic compounds: On the basis of the dehydratases of the two pathways to phenylalanine, five rRNA-DNA hybridization groups were distinguished. X. maltophilia was assigned to group V together with other Xanthomonas strains.

Group V could, however, not be distinguished from group Ib using these enzymes (BRADBURY, 1984).

Glycoside hydrolases: HAYWARD (1977) demonstrated the activity of α-glucosidase, β-glucosidase, β-galactosidase and β-xylosidase in most of the 39 Xanthomonas strains examined, as well as in X. maltophilia ("P". maltophilia) alone among the Pseudomonas species.

Cell walls: X. maltophilia and Xanthomonas share a high content of rhamnose, low content of 2-keto-3-deoxyoctonate, presence of D-galacturonic acid, and the absence of heptoses in their cell walls. All the Xanthomonas strains examined so far for the above characteristics, however, belonged only to X. campestris (BRADBURY, 1984). An interesting feature of the lipopolysaccharide of X. maltophilia is the presence of a pentose derivative that has been identified as 3-0-methyl-L-xylose, thus far not found in other bacteria with the exception of Rhodopseudomonas viridis (PALLERONI, 1984).

Exoproducts: A high degree of similarity (exceeding 90%) between the electrophoregrams of the exoproducts of 31 Pseudomonas strains, including (amongst others) X. maltophilia, P. aeruginosa, P. fluorescens, P. alcaligenes etc., has been shown by polyacrylamide gel electrophoresis (VOLCHKEVICH and DEGTEVA, 1989).

CONCLUSION

In conclusion, the following features are not inconsistent with inclusion of X. maltophilia in Xanthomonas: guanine-plus-cytosine contents, comparative enzymology, ubiquinones, cellular fatty acid composition, phage typing, oligonucleotide cataloging of 16S rRNA and glycoside hydrolase activity, although some features are not always as unique as implied. The following are, however, presently sufficient reasons to exclude X. maltophilia from Xanthomonas: the lack of xanthomonadins, xanthan gums and plant pathogenicity, its association with human infections, saprophytic survival in several niches, and its occurring free living in nature; also on the basis of the number of polar

flagella, the existence of pili or fimbriae, nitrate reduction, asparagine utilisation, low correlation in electrophoretic protein patterns, the number of properties shared also with Pseudomonas species (respiratory chain, exoproducts, the dehydratases of the two pathways to phenylalanine and growth factor requirement in some cases), the existence of methionine-nonrequiring strains of X. maltophilia, as well as several other differences mentioned, it does not seem appropriate or satisfactory to include X. maltophilia in Xanthomonas. DNA-DNA and DNA-rRNA hybridization results can be interpreted in various ways, but moreover also points to inclusion of the two taxons in a natural group as "close relatives", rather than inclusion in the same genus.

SWINGS et al. (1983) proposed an improved definition of the genus Xanthomonas, namely that "all Xanthomonas strains tested fall in the $T_{m(e)}$ range from 76 to 81°C when their DNAs are hybridized with labelled rRNA from type strain X. campestris pv. campestris NCPPB 528". It is, however, not clear to what extent this $T_{m(e)}$ range has been lowered mainly to include X. maltophilia.

The question of the reclassification of X. maltophilia is closely linked to the whole taxonomic situation in the genus Xanthomonas. Presently there are many arguments in favour of a phylogeneticaly based system of classification rather than a determinative one. It will, however, be a major setback for everyday phytobacteriology, where speed, cost effectiveness and the availability of sophisticated apparatus are often issues, if an improved definition of the genus Xanthomonas is based upon characters of phylogenetic value which can not be readily determined or repeated by less equipped laboratories. VAUTERIN et al. (1990) are for example of the opinion that in a general taxonomic environment there is no reason to assign crucial importance to a single phenotypic feature such as plant pathogenicity. Whether it is justified or not, plant pathogenicity and host specificity play a very important role in

the presumptive identification of xanthomonads by plant pathologists, and provision must be made for this feature, as well as several other practical means of differentiation, in the proposed improved definition of Xanthomonas. Although modern bacterial systematists can not be expected to work with an artificial type of classification for practical purposes only, a natural one without practical implication is also not acceptable (VAUTERIN et al., 1990). It is completely impracticable to define genera solely on the basis of phylogenetic data. Genera need to be characterised by using phenotypic properties, even if the choice of phenotypic markers might change given the development of better tests (MURRAY et al., 1990). The same applies to X. maltophilia. Although evidence of a more phylogenetic nature indicate a close relationship between X. maltophilia and Xanthomonas, its inclusion in Xanthomonas is not acceptable on practical grounds. In order to make provision for the natural similarities, as well as the significant differences discussed here, it is proposed that a separate genus is created for X. maltophilia, which could be placed with Xanthomonas in the same natural group.

REFERENCES

BRADBURY, J.F.: Genus II. Xanthomonas, pp. 199-120. In: Bergey's manual of systematic bacteriology (N.R. KRIEG, J.G. HOLT, eds.), 9th ed., vol. 1. Baltimore, Williams and Wilkens 1984

BYNG, G.S., WHITAKER, R.J., GHERNA, R.L., JENSEN, R.A.: Variable enzymological patterning in tyrosine biosynthesis as a new means of determining natural relatedness among Pseudomonadaceae. J. Bact. 144, 247-257 (1980)

DE LEY, J.: DNA base composition and hybridization in the taxonomy of phytopathogenic bacteria. Ann. Rev. Phytopath. 6, 63-90 (1968)

DE LEY, J., SEGERS, P., GILLIS, M.: Intra- and intergeneric similarities of Chromobacterium and Janthinobacterium ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 28, 154-168 (1978)

DE SMEDT, J., BAUWENS, M., TYTGAT, R., DE LEY, J.: Intra- and intergeneric similarities of ribosomal ribonucleic acid cistrons of free-living, nitrogen-fixing bacteria. Int. J. system. Bact. 30, 106-122 (1980)

DE SMEDT, J., DE LEY, J.: Intra- and intergeneric similarities of Agrobacterium ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 27, 222-240 (1977)

DE VOS, P., DE LEY, J.: Intra- and intergeneric similarities of Pseudomonas and Xanthomonas ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 33, 487-509 (1983)

DE VOS, P., GOOR, M., GILLIS, M., DE LEY, J.: Ribosomal ribocleic acid cistron similarities of phytopapthogenic Pseudomonas species. Int. J. system. Bact. 35, 169-184 (1985)

DYE, D.W.: The inadequacy of the usual determinative tests for the identification of Xanthomonas spp. N.S. J. Sci. 5, 393-416 (1962)

GARDNER, J.M., KADO, C.I.: Comparative base sequence homologies of the deoxyribonucleic acids of Erwinia species and other Enterobacteriaceae. Int. J. system. Bact. 22, 201-209 (1972)

GARRETT, C.M.E.: Bacterial diseases of food plants - an overview, pp. 115-132. In: Bacteria and plants (M.E. RHODES-ROBERTS, F.A. SKINNER, eds.). London, Academic Press 1982

GILARDI, G.L.: Characterization of Pseudomonas species isolated from clinical specimens. Appl. Microbiol. 21, 414-419 (1971)

GILLIS, M., DE LEY, J.: Intra- and intergeneric similarities of the ribosomal ribonucleic acid cistrons of Acetobacter and Gluconobacter. Int. J. system. Bact. 30, 7-27 (1980)

HAYWARD, A.C.: Occurrence of glycoside hydrolases in plant pathogenic and related bacteria. J. appl. Bact. 42, 407-411 (1977)

HUGH, R., RYSCHENKOW, E.: Pseudomonas maltophilia, an Alcaligenes-like species. J. gen. Microbiol. 26, 123-132 (1961)

IKEMOTO, S., SUZUKI, K., KANEKO, T., KOMAGATA, K.: Characterization of strains of Pseudomonas maltophilia which do not require methionine. Int. J. system. Bact. 30, 437-447 (1980)

JOHNSON, J.L.: Use of nucleic acid homologies in the taxonomy of anaerobic bacteria. Int. J. system. Bact. 23, 308-315 (1973)

JOHNSON, J.L., PALLERONI, N.J.: Deoxyribonucleic acid similarities among Pseudomonas species. Int. J. system. Bact. 39, 230-235 (1989)

KANDLER, O., SCHLEIFER, K-H.: Systematics of bacteria, pp. 234-237. In: Progress in botany (H. ELLENBERG, K., ESSER, K. KUBITZKI, E. SCHNEPF, H. ZIEGLER, eds.), vol. 42. Berlin, Springer-Verlag 1980

KOMAGATA, K., YABUUCHI, E., TAMAGAWA, Y., OHYAMA, A.: Pseudomonas melanogena Ilizuka and Komagata 1963, a later subjective synonym of Pseudomonas maltophilia Hugh and Ryschenkow 1960. Int. J. system. Bact. 24, 242-247 (1974)

MURRAY, R.G.E., BRENNER, D.J., COLWELL, R.R., DE VOS, P., GOODFELLOW, M., GRIMONT, P.A.D., PFENNIG, N., STACKEBRANDT, E., ZAVARZIN, G.A.: Report of the ad hoc committee on approaches to taxonomy within the proteobacteria. Int. J. system. Bact. 40, 213-215 (1990)

OYAIZU, H., KOMAGATA, K.: Grouping of Pseudomonas species on the basis of cellular fatty acid compositions and the quinone system with special reference to the existence of 3-hydroxy fatty acids. J. gen. appl. Microbiol. 29, 17-40 (1983)

PALERONI, N.J.: Introduction to the family Pseudomonadaceae, pp. 655-665. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (M.P. STARR, H., STOLP, H.G. TRÜPER, A. BARLOWS, H.G. SCHLEGEL, eds.). Berlin, Springer-Verlag 1981

PALLERONI, N.J.: Family I. Pseudomonadaceae, pp. 141-199. In: Bergey's manual of systematic bacteriology (N.R. KRIEG, J.G. HOLT, eds.), 9th ed., vol. I. Baltimore, Williams and Wilkins 1984.

PALLERONI, N.J.: Taxonomy of the pseudomonads. pp. 3-25. In: The bacteria: the biology of Pseudomonas (J.R. SOKATCH, ed.). London, Academic Press 1986

PALLERONI, N.J., KUNISAWA, R., CONTOPOULOU, R., DOUDOROFF, M.: Nucleic acid homologies in the genus Pseudomonas. Int. J. system. Bact. 23, 333-339 (1973)

ROBBS, C.F., RIBEIRO, R. de L.D., KIMURA, O., AKIBA, F.: Variaqoes em Xanthomonas manihotis (Arthaud-Berthet) Sstarr. Rev. Soc. Bras. Fitopatol. 5, 67-75 (1972)

ROBBS, C.F., RIBEIRO, R. de L.D., KIMURA, O.: Sobre a posiqao taxonomica de Pseudomonas mangiferaeindicae Patel et al. 1948, agente causal da "mancha bacteriana" das folhas da mangueira (Mangifera infica L.). Arq. Univ. Fed. Rural Rio de Janeiro 4, 11-14 (1974)

SABET, K.A.: Studies in the bacterial diseases of Sudan crops. II. Bacterial leaf blight disease of castor (Ricinus communis L.). Ann. appl. Biol. 47, 49-56 (1959)

STARR. M.P.: The nutrition of phytopathogenic bacteria. I. Minimal requirements of the genus Xanthomonas. J. Bact. 51, 131-143 (1946)

STARR, M.P.: The genus Xanthomonas, pp. 742-763. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (M.P. STARR, H. STOLP, H.G. TRÜPER, A., BARLOWS, H.G. SCHLEGEL, eds.). Berlin, Springer-Verlag 1981

STARR, M.P., STEPHENS, W.L.: Pigmentation and taxonomy of the genus Xanthomonas. J. Bact. 87, 293-302 (1964)

SWINGS, J., DE VOS, P., VAN DEN MOOTER, M., DE LEY, J.: Transfer of Pseudomonas maltophilia Hugh 1981 to the genus Xanthomonas as Xanthomonas maltophilia (Hugh 1981) comb. nov. Int. J. system. Bact. 33, 409-413 (1983)

VAN ZYL, E., STEYN, P.L.: Differentiation of phytopathogenic Pseudomonas and Xanthomonas species and pathovars by numerical taxonomy and protein gel electrophoregrams. System. Appl. Microbiol. 13, 60-71 (1990)

VAUTERIN, L., SWINGS, J., KERSTERS, K., GILLIS, M., MEW, T.W., SCHROTH, M.N., PALLERONI, N.J., HILDEBRAND, D.C., STEAD, D.E., CIVEROLO, E.L., HAYWARD, A.C., MARAÎTE, H., STALL, R.E., VIDAVER, A.K., BRADBURY, J.F.: Towards an improved taxonomy of Xanthomonas. Int. J. system. Bact. 40, 312-316 (1990)

VOLCHKEVICH, Z.A., DEGTEVA, G.K.: Electrophoretic analysis of Pseudomonas exoproducts. Zh. Mikrobiol. Epidemiol. Immunobiol. 8, 24-28 (1989)

WAYNE, L.G., BRENNER, D.J., COLWELL, R.R., GRIMONT, P.A.D., KANDLER, O., KRICHEVSKY, M.I., MOORE, L.H., MOORE, W.E.C., MURRAY, R.G.E., STACKEBRANDT, E., STARR, M.P., TRÜPER, H.G.: Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. system. Bact. 37, 463-464 (1987)

WHITAKER, R.J., BYNG, G.S., GHERNA, R.L., JENSEN, R.A.: Comparative allostery of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase as an indicator of taxonomic relatedness in pseudomonad genera. J. Bact. 145, 752-759 (1981)

WOESE, C.R., BLANZ, P., HAHN, C.M.: What isn't a pseudomonad: the importance of nomenclature in bacterial classification. System. Appl. Microbiol. 5, 179-195 (1984)

CHAPTER V

ISOLATION OF <u>XANTHOMONAS</u> <u>CAMPESTRIS</u> PV. <u>MANGIFERAEINDICAE</u> FROM GALL FLY-INDUCED LESIONS ON MANGO LEAVES

SUMMARY

A bacterium was isolated from gall lesions on mango leaves caused by the gall fly Procontarinia matteiana. By means of morphological, physiological, biochemical and pathogenicity tests and polyacrylamide gel electrophoresis, the isolate was identified as Xanthomonas campestris pv. mangiferaeindicae, the causal organism of bacterial black spot of mangoes.

INTRODUCTION

In 1986 a sudden increase in gall lesions on mango leaves caused by the gall fly Procontarinia matteiana, occurred at the Lisbon Estate in the Eastern Transvaal. Of particular interest was the association of bacterial black spot-like lesions with most of these galls. No previous reference to this type of association could be found in the literature. It was speculated that these lesions in fact could have been bacterial black spot, although it was not clear whether the pathogen entered the wound by chance or whether it was transmitted by the insect.

The purpose of this study was therefore to determine whether the bacterial black spot pathogen, Xanthomonas campestris pv. mangiferaeindicae, or any other plant pathogenic bacteria could be isolated from these gall lesions, and to test the applicability of PAGE to practical phytobacteriology.

MATERIALS AND METHODS

Isolations were made from the gall lesions after surface disinfestation with 0,1% NaOCl for 60 s. Watersoaked areas of the black spot-like lesions were cut out with a scalpel and plated directly onto GYCA medium (VERA CRUZ et al., 1984). Alternatively, these lesions were soaked in sterile distilled water for 1 h, after which the fluid was streaked onto GYCA plates. As most of the developing growth appeared identical, two colonies, chosen at random, were purified by repeated streaking and maintained on GYCA medium for further study. These two

strains were included in the morphological, physiological and biochemical tests (DYE, 1962; HARRIGAN and McCANCE, 1966; HAYWARD, 1966; HENDRIE and SHEWAN, 1966; STEYN et al., 1974; MANICOM and WALLIS, 1984) that were being performed on a wide variety of Xanthomonas and plant pathogenic Pseudomonas spp. (VAN ZYL and STEYN, 1990). Data were analysed using the simple matching (S_{SM}) coefficient and unweighted pair-group clustering (SNEATH and SOKAL, 1973).

The discontinuous PAGE system of LAEMMLI (1970) was used to compare the unknown strains electrophoretically with a reference strain of X. campestris pv. mangiferaeindicae (NCPPB 490). Normalized scans were analysed as described by KERSTERS and DE LEY (1975).

Pathogenicity was tested in the greenhouse on young mango trees (Kent and Peach cultivars). Trees were predisposed by spraying with water for 48 h, after which leaves were surface disinfested and lightly wounded with a sharp, sterile needle. The inoculum, standardised spectrocolorometrically to a density of 10^8 cells cm^{-3} was applied onto the wound in an aqueous suspension. The trees were then enclosed in transparent plastic bags for 24 h (STEYN et al., 1974). Greenhouse temperatures ranged from 20-30°C.

RESULTS

Examination of the bacterial cultures revealed Gram-negative, nonsporulating rods, motile by means of a single polar flagellum as revealed by transmission electron microscopy of negatively stained preparations. Round, shallow convex colonies with entire margins were formed on GYCA medium. The colour was initially smoke grey but soon became white to cream. Copious slime was produced. No xanthomonadin or fluorescent pigments were produced.

Computer-assisted analysis of physiological and biochemical tests, showed that these two unknown strains formed a tight cluster with the rest of the X. campestris group that was used in this study. A similarity of 95,8% S_{SM} was obtained between the unknown strains and the black spot reference strain (NCPPB 490). The Pseudomonas group clustered separately (VAN ZYL and STEYN, 1990).

There was no noticeable difference between the electro-phoregrams of the unknown strains and the black spot reference strain when compared visually. This observation was confirmed by quantitative computer-assisted clustering that produced a clustering level of r = 0,96 for the unknown strains and the reference strain.

Both strains caused typical bacterial black spot symptoms on mango leaves. The pathogen was reisolated on GYCA medium.

DISCUSSION

Morphological, physiological and biochemical characteristics of the strains studied, as well as the result of electrophoresis and pathogenicity tests are considered sufficient evidence to establish the identity of the bacterium isolated from gall lesions on mango leaves, as X. campestris pv. mangiferaeindicae, the causal agent of bacterial black spot of mangoes. The effectiveness of PAGE as a method for the rapid identification and grouping of unknown organisms (KERSTERS and DE LEY, 1980) was illustrated by the fact that the unknown strains in this study were positively identified within 4 d of isolation by means of electrophoresis, in comparison with 14 and 42 d respectively for pathogenicity and biochemical tests. This is also the first report that X. campestris pv. mangiferaeindicae was found in association with the gall lesions caused by the mango leaf gall fly. As the disease can also spread without the conspicuous presence of the insect, the association between the pathogen and

the gall fly may be incidental and validates further investigation.

REFERENCES

DYE, D.W.: The inadequacy of the usual determinative tests for the identification of Xanthomonas spp. N.Z. J. Sci. 5: 393-416 (1962)

HARRIGAN, W.F., McCANCE, M.E.: Laboratory methods in microbiology. London, Academic Press 1966

HAYWARD, A.C.: Methods of identification in the genus Xanthomonas pp. 9-14. In: Identification methods for microbiologists (B.M. GIBBS and G.A. SKINNER, eds.), part A. London, Academic Press 1966

HENDRIE, M.S., SHEWAN, J.M.: The identification of certain Pseudomonas species, pp. 1-7. In: Identification methods for microbiologists (B.M. GIBBS and F.A. SKINNER, eds.), part A. London, Academic Press 1966

KERSTERS, K., DE LEY, J.: Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. J. gen. Microbiol. 87: 333-342 (1975)

KERSTERS, K., DE LEY, J.: Classification and identification of bacteria by electrophoresis of their proteins, pp. 273-297. In: Microbiological classification and identification (M. GOODFELLOW & R.G. BOARD, eds.). London, Academic Press 1980

LAEMMLI, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680-685 (1970)

MANICOM, B.Q., WALLIS, F.M.: Further characterization of Xanthomonas campestris pv. mangiferaeindicae. Ann. appl. Biol. 109: 129-135 (1984)

SNEATH, P.H.A., SOKAL, R.R.: Numerical taxonomy: The principles and practice of numerical classification. San Francisco, W.H. Freemand and Co 1973.

STEYN, P.L., VILJOEN, N.M., KOTZé, J.M.: The causal organism of bacterial black spot of mangoes. Phytopath. 64: 1400-1404 (1974)

VAN ZYL, E., STEYN P.L.: Differentiation of phytopathogenic Pseudomonas and Xanthomonas species and pathovars by numerical taxonomy and protein gel electrophoregrams. System. Appl. Microbiol. 13: 60-71 (1990)

VERA CRUZ, C.M., GOSSELé, F., KERSTERS, K., SEGERS, P., VAN DEN MOOTER, M., SWINGS, J., DE LEY, J.: Differentiation between Xanthomonas campestris pv. oryzae, Xanthomonas campestris pv. oryzicola and the bacterial "Brown blotch" pathogen on rice by numerical analysis of phenotypic features and protein gel electrophoregrams. J. gen. Microbiol. 130: 2983-2999 (1984)

CHAPTER VI

SYNOPSIS

GENERAL CONCLUSIONS

From this study, it can be concluded that SDS-PAGE of the total soluble proteins of the bacterial cell is a powerful, rapid and relatively simple method, generally applicable for the differentiation and identification of phytopathogenic Pseudomonas and Xanthomonas species and pathovars.

The clustering obtained from numerical analysis of protein electrophoregrams agrees favourably with that obtained from classical phenotypic features, as well as phylogenetic groupings based on DNA-DNA/DNA-rRNA homology, as gathered from literature. Polyacrylamide gel electrophoresis of cell proteins will therefore be a reliable taxonomic marker in a polyphasic approach to supplement and evaluate phylogenetic relationships.

Polyacrylamide gel electrophoresis of cell proteins to a certain extent fulfils the requirements of phylogenetic taxonomy, in being an objective, high-resolution, molecular technique, correlating well with DNA homology. Furthermore, in the case of the phytopathogenic bacteria, it is not subjectively influenced by phytopathogenicity or host specificity. On the other hand, this technique also meets the demands of phytobacteriology, by being relatively easy to perform, rapid and sensitive, allowing even visual differentiation and grouping of pathovars. It furthermore may allow differentiation of phytopathogenic and saprophytic species, as illustrated in this study by the P. aeruginosa/P. fluorescens group and Comamonas acidovorans.

The subgeneric relationships determined by classical phenotypic features, as well as protein electrophoregrams, within rRNA homology groups I, II, III and V, should contribute to the proposed polyphasic approach and progress in Pseudomonas and Xanthomonas taxonomy. PAGE also contributed to determining the homogeneity or heterogeneity of the taxa involved (e.g. the P. solanacearum, P. avenae and X. campestris groups). Misnamed strains (e.g. P. avenae (NCPPB 2402)), strains wrongly thought to

be synonomous with other accepted species (e.g. P. angulata (ATCC 13453) and "P. setariae" (NCPPB 1392)), and classification of strains of uncertain affinity (e.g. P. caricapapayae and P. tolaasii) were detected.

Whereas modern bacterial systematists cannot be expected to work with an artificial type of classification for practical purposes only, a natural system without practical implications that might be of little use to the microbiologist in the field, is also not acceptable. The inclusion of X. maltophilia in the genus Xanthomonas primarily on phylogenetic grounds, is therefore questioned. As a result of this study it is believed that phylogenetic data alone are insufficient to provide an adequate and workable description of genera, and that coherent phenotypic characteristics are essential for both description and recognition.

The rapid identification of the bacterium found in association with the gall lesions on mango leaves, is an example of the applicability of PAGE to common phytobacteriological practice. Once electrophoretic groups of reliable taxonomic status have been delineated, unknown bacteria can be compared with them fairly quickly for eventual identification, thus eliminating time-consuming procedures such as the performance of a large number of phenotypic tests or the inoculation of a range of possible hosts. This will be particularly helpful in the case of strains of unknown origin or from a previously unreported plant disease, avirulent strains, and nonpigmented xanthomonads.

This study has revealed further advantages of visual or computer-assisted comparison of electrophoretic protein patterns which can be of value to a polyphasic approach as well as phytobacteriology. Large numbers of cultures can be screened and grouped into identical or closely related strains, so as to limit identification procedures or DNA hybridizations to representative strains of each group only. As DNA homology will in future be the standard arbiter for the designation of species (MURRAY et

al., 1990), PAGE can be used to compare newly isolated strains with DNA reference strains relatively easily and fast, as strains with a high genome DNA similarity display very similar or almost identical protein patterns (KERSTERS and DE LEY, 1980). Visual comparison of electrophoretic patterns eliminates uncertainty due to variation in colony type (such as between virulent and avirulent strains of P. solanacearum).

Computer-assisted numerical analysis, both of classical phenotypic features, as well as electrophoretic data, for comparing, clustering, storage and retrieval of information, was found to be of great value.

The limitations and demands of the PAGE technique must be realized in order to avoid unrealistic expectations. In order for results to be reproducible, rigorously controlled and standardised conditions and procedures have to be followed. Comparison of protein profiles is only possible for bacteria grown on identical media under strictly reproducible conditions. Better comparisons are made between protein profiles on the same gel or gels run together, than in samples separated in different electrophoretic runs. The sensitivity of the densitometric analysis is very important and has to be tested and standardised by using several controls. Although the method is relatively easy and fast, manual normalization and measuring of scans are tedious and time-consuming.

In the final analysis, good scientific judgement in the light of other knowledge is indispensible for interpreting results of numerical taxonomy (SNEATH, 1986).

REFERENCES

KERSTERS, K., DE LEY, J.: Classification and identification of bacteria by electrophoresis of their proteins, pp. 273-297. In: Microbiological classification and identification (M. GOODFELLOW, R.G. BOARD, eds.). London, Academic Press 1980

MURRAY, R.G.E., BRENNER, D.J., COLWELL, R.R., DE VOS, P., GOODFELLOW, M., GRIMONT, P.A.D., PFENNIG, N., STACKEBRANDT, E., ZAVARZIN, G.A.: Report of the ad hoc committee on approaches to taxonomy within the proteobacteria. Int. J. system. Bact. 40, 213-215 (1990)

SNEATH, P.H.A.: Numerical taxonomy, pp. 969-971. In: Bergey's manual of systematic bacteriology (P.H.A. SNEATH, N.S. MAIR, M.E. SHARPE, J.G. HOLT, eds.), 9th ed., vol. 2. London, Williams and Wilkins 1986

ADDENDUM

This section includes the papers that appeared or were accepted for publication in <u>Systematic and Applied Microbiology</u> and <u>Phytophylactica</u>. One manuscript was submitted for publication to <u>Systematic and Applied Microbiology</u>.

Reprint from

SYSTEMATIC AND APPLIED MICROBIOLOGY

RESEARCH NOTE

ISOLATION OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* FROM GALL FLY-INDUCED LESIONS ON MANGO LEAVES

ELRITHA VAN ZYL, J. M. KOTZÉ and P. L. STEYN, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0001

ABSTRACT

Key words: Mango leaf gall fly, bacterial black spot, *Xanthomonas campestris* pv. *mangiferaeindicae*, polyacrylamide gel electrophoresis

A bacterium was isolated from gall lesions on mango leaves caused by the gall fly *Procontarinia matteiana*. By means of morphological, physiological, biochemical and pathogenicity tests and polyacrylamide gel electrophoresis, the isolate was identified as *Xanthomonas campestris* pv. *mangiferaeindicae*, the causal organism of bacterial black spot of mangoes.

Uittreksel

ISOLASIE VAN XANTHOMONAS CAMPESTRIS PV. MANGIFERAEINDICAE *VANUIT GALVLIEG-GEÏNDUSEERDE LETSELS OP MANGOBLARE*

'*n Bakterie is geïsoleer vanuit galletsels op mangoblare wat veroorsaak is deur die galvlieg* Procontarinia matteiana. *Hierdie bakterie-isolaat is met behulp van morfologiese, fisiologiese, biochemiese en patogenisiteitstoetse en poli-akrielamied-jel-elektroforese geïdentifiseer as* Xanthomonas campestris *pv.* mangiferaeindicae, *die veroorsakende agens van bakteriese swartvleksiekte van mango's.*

In 1986 a sudden increase in gall lesions on mango leaves caused by the gall fly *Procontarinia matteiana* occurred at the Lisbon Estate in the Eastern Transvaal. Of particular interest was the association of bacterial black spot-like lesions with most of these galls. No previous reference to this type of association could be found in the literature. It was speculated that these lesions could, in fact, have been bacterial black spot, although it was not clear whether the pathogen entered the wound by chance or whether it was transmitted by the insect.

The purpose of this study was therefore to determine whether the bacterial black spot pathogen or any other plant pathogenic bacteria could be isolated from these gall lesions.

Isolations were made from the gall lesions after surface disinfestation with 0,1 % NaOCl for 60 s. As most of the developing colonies appeared identical, two were chosen at random and purified by repeated streaking and maintained on GYCA medium (Cruz *et al.*, 1984) for further study. These two strains were included in the morphological, physiological and biochemical tests (Dey, 1962; Harrigan & McCance, 1966; Hayward, 1966; Hendrie & Shewan, 1966; Steyn, Viljoen & Kotzé, 1974; Manicom & Wallis, 1984) that were being performed on a wide variety of *Xanthomonas* and plant pathogenic *Pseudomonas* spp. (paper in preparation). Data were analysed using the simple matching (S_{sm}) coefficient and unweighted pair-group clustering (Sneath & Sokal, 1973).

The discontinuous PAGE system of Laemmli (1970) was used to compare the unknown strains electrophoretically with a reference strain of *X. campestris* pv. *mangiferaeindicae* (NCPPB 490). Normalized scans were analysed as described by Kersters & De Ley (1975).

Pathogenicity was tested in the greenhouse on young mango trees (cv. Kent and Peach). Trees were predisposed by spraying with water for 48 h, after which leaves were surface disinfested and lightly wounded with a sharp, sterile needle. The inoculum, standardised spectrocolorometrically to a density of 10^8 cells cm^{-3} was applied onto the wounds in an aqueous suspension. The trees were then enclosed in transparent plastic bags for 24 h (Steyn *et al.*, 1974). Greenhouse temperatures ranged from 20–30 °C.

Examination of the bacterial cultures revealed Gram-negative, nonsporulating rods, motile by means of a single polar flagellum as revealed by transmission electron microscopy of negatively stained preparations. Round, shallow-convex colonies with entire margins were formed on GYCA medium. The colour was initially smoke grey but soon became white to cream. Copious slime was produced. No xanthomonadin or fluorescent pigments were produced.

Computer-assisted analysis of physiological and biochemical tests showed that these two unknown strains formed a tight cluster with the rest of the *X. campestris* group that was used in this study. A similarity of 95,8 % S_{sm} was obtained between the unknown strains and the black spot reference strain (NCPPB 490). The *Pseudomonas* group clustered separately (paper in preparation).

There was no noticeable difference between the electrophoregrams of the unknown strains and the black spot reference strain when compared visually. This observation was confirmed by quantitative computer-assisted clustering that produced a clustering level of r = 0,96 for the unknown strains and the reference strain.

Both strains caused typical bacterial black spot symptoms on mango leaves. The pathogen was reisolated on GYCA medium.

Morphological, physiological and biochemical characteristics of the strains studied, as well as the result of electrophoresis and pathogenicity tests are considered sufficient evidence to establish the identity of the bacterium isolated from gall lesions on mango leaves as *X. campestris* pv. *mangiferaeindicae*, the causal agent of bacterial black spot of mangoes. The effectiveness of PAGE as a method for the rapid identification and grouping of unknown organisms (Kersters & De Ley, 1980) was illustrated by the fact that the unknown strains in this study were positively identified within 4 d of isolation by means of electrophoresis, in comparison with 14 and 42 d respectively for pathogenicity and biochemical tests. This is also the first report that *X. campestris* pv. *mangiferaeindicae* is found in association with the gall lesions caused by the mango leaf gall fly. As the disease can also spread without the conspicuous presence of the insect, the association between the pathogen and the gall fly might be incidental and validates further investigation.

ACKNOWLEDGEMENTS

REFERENCES

CRUZ, C. M. V., GOSSELE, F., KERSTERS, K., SEGERS, P., VAN DEN MOOTER, M., SWINGS, J. & DE LEY, J., 1984. Differentiation between *Xanthomonas campestris* pv. *oryzae, Xanthomonas campestris* pv. *oryzicola* and the bacterial "Brown blotch" pathogen on rice by numerical analysis of phenotypic features and protein gel electrophoregrams. *Journal of General Microbiology* 130: 2983–2999.

DEY, D. W., 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. *New Zealand Journal of Science* 5: 393–416.

HARRIGAN, W. F. & MCCANCE, M. E., 1966. Laboratory methods in microbiology. Academic Press, London & New York.

HAYWARD, A. C., 1966. Methods of identification in the genus *Xanthomonas*. Pages 9–14 in: Identification methods for microbiologists. Part A., B. M. Gibbs & F. A. Skinner, eds. Academic Press, London & New York.

HENDRIE, M. S. & SHEWAN, J. M., 1966. The identification of certain *Pseudomonas* species. Pages 1–7 in: Identification methods for microbiologists. Part A., B. M. Gibbs & F. A. Skinner, eds. Academic Press, London & New York.

KERSTERS, K. & DE LEY, J., 1975. Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. *Journal of General Microbiology* 87: 333–342.

KERSTERS, K. & DE LEY, J., 1980. Classification and identification of bacteria by electrophoresis of their proteins. Pages 273–297 in: Microbiological classification and identification. M. Goodfellow & R. G. Board, eds. Academic Press. London & New York.

LAEMMLI, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227: 680–685.

MANICOM, B. Q. & WALLIS, F. M., 1984. Further characterization of *Xanthomonas campestris* pv. *mangiferaeindicae. Annals of Applied Biology* 109: 129–135.

SNEATH, P. H. A. & SOKAL, R. R., 1973. Numerical taxonomy: The principles and practice of numerical classification. W. H. Freeman & Co., San Francisco.

STEYN, P. L., VILJOEN, N. M. & KOTZÉ, J. M., 1974. The causal organism of bacterial black spot of mangoes. *Phytopathology* 64: 1400–1404.

Differentiation of Phytopathogenic *Pseudomonas* and *Xanthomonas* Species and Pathovars by Numerical Taxonomy and Protein Gel Electrophoregrams

ELRITHA VAN ZYL and P. L. STEYN

Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, Republic of South Africa

Summary

The applicability of numerical analysis of polyacrylamide gel electrophoregrams of whole cell protein extracts for the classification and rapid identification of phytopathogenic *Pseudomonas* and *Xanthomonas* species and pathovars was investigated and compared with numerical analysis of classical phenotypic features. It is concluded that electrophoresis of the total soluble proteins of the bacterial cell is a powerful, rapid and relatively easy method, applicable to everyday use, for the differentiation of phytopathogenic pseudomonads and xanthomonads up to a sub-specific level. Results correlated to a high degree with the clustering obtained from classical phenotypic features. On the basis of this study several taxonomic proposals, concerning this group of organisms, are made.

Introduction

The genera *Pseudomonas* Migula 1894 and *Xanthomonas* Dowson 1939 are of substantial economic importance, because members of these genera constitute more than three-quarters of all phytopathogenic bacteria (*Stolp* et al., 1965). The taxonomy of these phytopathogens and their interrelationships are not yet fully resolved. Consequently, practical difficulties are frequently encountered in their routine classification and identification (*Palleroni*, 1981; *Schroth* et al., 1981; *Starr*, 1981; *De Vos* and *De Ley*, 1983; *Oyaizu* and *Komagata*, 1983; *Palleroni*, 1984; *De Vos* et al., 1985).

The present generic definitions of *Pseudomonas* and *Xanthomonas* do not allow a sharp separation between them (*De Vos* et al., 1985). Some members of these two genera actually share a substantial degree of DNA-rRNA homology, and therefore unequivocal allocation to either one of these genera is difficult for some isolates (*Palleroni*, 1984; *De Vos* et al., 1985).

Solid bacteriological criteria for the differentiation of *Pseudomonas* species and pathovars from one another and from saprophytic forms are still needed and, consequently, strains isolated from sources other than plant lesions seldom can be assigned with certainty to described species or pathovars (*Palleroni*, 1984).

One technique of classification and identification at a molecular level is the relatively simple and inexpensive method of polyacrylamide gel electrophoresis of the total soluble proteins of the bacterial cell (*El-Sharkawy* and *Huisingh*, 1971; *Palmer* and *Cameron*, 1971; *Kersters* and *De Ley*, 1980; *Starr*, 1981; *Vera Cruz* et al., 1984; *Van den Mooter* et al., 1987). When used to screen isolates, this procedure reduced total analytical time and expense without sacrificing accuracy (*Moore* et al., 1980, *Krieg* and *Jones*, 1986). Results of this method also correlate well with those of DNA-DNA hybridizations (*Kersters* and *De Ley*, 1980; *Jackman*, 1982; *Owen* and *Jackman*, 1982) and numerical analysis of phenotypic features (*Kersters* and *De Ley*, 1975; *Vera Cruz* et al., 1984).

In this study numerical analysis of polyacrylamide gel electrophoregrams of whole cell protein extracts was used for the routine differentiation and identification of phytopathogenic *Pseudomonas* and *Xanthomonas* species and

pathovars. The results thus obtained were compared with those obtained from numerical analysis of classical phenotypic features of the same group of organisms.

Materials and Methods

Bacterial strains. A list of the bacteria used in the study is given in Table 1. Two colony types were found in four of the *Pseudomonas solanacearum* strains and in *Xylophilus ampelinus*. As a preliminary investigation of the *P. solanacearum* strains showed no difference in the electrophoretic protein patterns of the different colony types, only the white, mucoid, virulent type (*Buddenhagen* and *Kelman*, 1964) from each strain was used for the phenotypic tests. In the case of *Xy. ampelinus*, the two types differed in growth rate, with only the slow growing colony type corresponding to the description of *Xy. ampelinus* (*Willems* et al., 1987). Therefore, only this strain was used for the phenotypic tests. However, the slow-growing strain could not be mass-cultured in broth medium successfully for electrophoresis. The fast-growing strain of *Xy. ampelinus*, however, grew satisfactorily in this medium, producing a mucoid, light-yellow type of growth very similar to that of *X. axonopodis*. UP 89 was isolated from bacterial black spot of mangoes and identified as "*P. mangiferae-indicae*" (*Steyn* et al., 1974). One of the *X. campestris* pv. *mangiferaeindicae* strains (D42) was a local isolation from gall lesions on mango leaves (*Van Zyl* et al., 1988).

Culture conditions. The bacteria were grown at 28 °C on GYCA medium (*Vera Cruz* et al., 1984). With the exception of *P. solanacearum* strains, that were maintained in sterile water at room temperature (15–25 °C), all were maintained on GYCA slants, also at room temperature. Strains were transferred to fresh medium every 2 to 3 weeks and checked for purity by plating and by microscopic examination of living and Gram-stained cells. All isolates were also freeze-dried.

For electrophoretic studies cultures were fully grown in 200 ml Std 1 nutrient broth (Merck), supplemented with 0.15% K_2HPO_4 and 0.15% $MgSO_4.7H_2O$, while being shaker-incubated at 28 °C.

Morphological, physiological and biochemical features. If not otherwise stated, all physiological tests were performed according to *Dye* (1962). Carbon sources were added aseptically from filter-sterilized, concentrated solutions. Cultures were examined for growth and acid production after 2, 4 and 7 d, then at 7-day intervals to 42 d.

Kovacs' (1956) reagent was used for the oxidase test. The production of a fluorescent pigment was tested on King, Ward and Raney's medium B (*Harrigan* and *McCance*, 1966). The oxidation-fermentation (O/F) test was performed according to *Hugh* and *Leifson* (1953), using glucose as carbon source. Hydrolysis of Tween 80 was tested according to *Sierra* (1957). β-Galactosidase activity was detected by the ONPG test (*Hayward*, 1977). Hydrolysis of arginine with the formation of ammonia was tested on *Thornley's* semi-solid arginine medium (1960). Protein digestion in liquid and agar medium was performed according to *Dye* (1980), using "purple milk" and milk agar plates. Poly- β-hydroxybutyrate accumulation was observed by phase contrast microscopy of Sudan black B stained smears according to *Sands* et al. (1980). Xanthomonadin production was observed visually on GYCA medium, and results were confirmed from *Bradbury* (1984).

The suspensions used for the inoculation of all media were prepared from 48 h GYCA cultures and were standardised spectrocolorometrically to approximately 10^7 cells/ml. Liquid media were inoculated by the addition of two drops from a sterile 1-ml pipette, while one loopful of the suspension was streaked on solid media. Unless otherwise stated, incubation times were as specified by the different authors. Incubation temperature was always 28 °C except for gelatinase (25 °C). All liquid media were shaker incubated.

One strain each of *Eschericia coli* and *Bacillus cereus* was used as a control.

Numerical analysis of phenotypic features. Fifty-three phenotypic features were determined for each of the 38 strains. All phenotypic features were coded as 1 (positive) or 0 (negative). There were no missing features and dubious results were repeated. Similarity coefficients (S_{SM}) (*Sokal* and *Michener*, 1958) were calculated and the strains were clustered by unweighted average linkage (*Sneath* and *Sokal*, 1973) using a program in Basic written by Dr. *J. J. Bezuidenhout* (Univ. of Pretoria) and an IBM Personal Computer.

Polyacrylamide gel electrophoresis of soluble proteins
Preparation of whole cell extracts.

Cells were harvested from fully grown cultures by centrifugation, washed in sterile, distilled water and centrifuged again. Cultures were checked for purity at harvest by microscopy and streaking on GYCA plates. Harvested cells were stored at −12 °C in sterile screwcap bottles (vol. 20 ml).

When ready to commence with elecrophoresis, 0.5 g (wet weight) of cells from each strain was resuspended in 15 ml sample buffer (*Laemmli*, 1970) and sonicated with a Dawe soniprobe (Type 7530A) with an 80 watt output for 2 min in 20 s periods, alternating with 5 to 8 s pauses for cooling.

Throughout, the cell suspensions were submerged in ice to inhibit proteases. The sonicated suspensions were then heated for 2 min in a boiling waterbath to ensure denaturation of the proteins. Thereafter, 1.5 ml of the crude sonicate was centrifuged for 2 min in a bench-top centrifuge to remove the cellular debris and mucus which originated from the slimy growth of some strains. The final supernatant contained the total soluble proteins used for electrophoresis. Protein concentrations of the supernatants were standardised according to *Stegemann* et al. (1987). Prepared samples were used as soon as possible. When unavoidable, samples were stored at −12 °C.

Standard conditions for polyacrylamide gel electrophoresis. The discontinuous sodium dodecyl sulphate (SDS) buffer system of *Laemmli* (1970) was used as a basis for one dimensional slab gel electrophoresis in a Protean II vertical electrophoresis unit (Bio-Rad). Preparation, casting, assembling and running of the gels were performed according to the Protean II Slab Cell Instruction Manual (Bio-Rad, 1984), using a 10% separating gel (pH 8.8) and a 4% stacking gel (pH 6.8). Gels were 1.5 mm thick and 160 mm long. The final concentration of SDS was 0,1% in both gels. A Tris glycine electrode buffer (pH 8.3) was used. All buffers and stock solutions were stored at 4 °C but used at room temperature. Gels were cast one day prior to the electrophoretic run and left overnight at room temperature to ensure complete polymerization.

Fourteen samples (containing approx. 100 µg of soluble protein/20 µl each) (*Hames*, 1981) were loaded into 14 of the 15 sample wells on one gel with a Hamilton syringe. One well was loaded with the reference proteins, namely 10 µl bovine thyroglobulin (mol wt: 669000; Type I, Sigma; 0.01 g/ml) and 5 µl ovalbumin (mol wt: 43000; Grade V, Sigma; 0.01 g/ml) suspended in sample buffer. Five µl lysozyme (mol wt: 14300; 0.01 g/10 ml sample buffer) was added to each well as a bottom reference point on all the electrophoregrams. These reference proteins enabled accurate comparisons between protein patterns on different gels. A bromophenol blue solution containing 50% (v/v) glycerol was added as a tracking dye during electrophoresis. Two gels were run simultaneously. Electrophoresis of the proteins of all the strains was performed repeatedly until satisfactory and reproducible results were obtained.

Table 1. Strains used for phenotypic tests and protein gel electrophoresis
a) ATCC, American Type Culture Collection, Rockville, Maryland;
NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, England;
PDDCC, Culture Collection of the Plant Disease Division, Department of Scientific and Industrial Research, Auckland, New Zealand;
TCRI, Tobacco and Cotton Research Institute, Kroondal, South Africa;
VOPRI, Vegetable and Ornamental Plant Research Institute, Pretoria, South Africa.
b) Omitted from the Approved Lists of Bacterial Names (*Skerman* et al., 1981).
c) *P. marginalis* pv. *marginalis*; not included in electrophoretic studies.
d) *P. marginalis* pv. *alfalfae*.
e) Two colony types were found for these strains. Only the irregularly-round, white, fluidal colonies were used for phenotypic tests, while both the white, mucoid type (t1) and the butyrous, brown type (t2) were used for electrophoresis.
f) Supplied by *M. C. Engelbrecht*, TCRI, S.A.
g) Supplied by *A. Swanepoel*, VOPRI, S.A.
h) Supplied by Dr. *M. L. Moffett*, Queensland, Australia.
i) Isolated in 1972 by *N. Viljoen*.
j) Formerly *Xanthomonas ampelina*. Reclassified as *Xylophilus ampelinus* by *Willems* et al. (1987).

PHENON	STRAIN NO[a]	SPECIES/PATHOVAR NAME	OTHER STRAIN DESIGNATIONS	HOST PLANT	LOCALITY OF ORIGIN (IF NOT FROM CULTURE COLLECTION)
I	NCPPB 2653	P. aeruginosa	ATCC 10145		
	ATCC 13453	P. angulata[b]		Nicotiana tabacum	
	NCPPB 1011	P. avenae	ATCC 19860	Avena sativa	
	NCPPB 1873	P. caricapapavae		Carica papava	
	NCPPB 1964	P. fluorescens bv. I	ATCC 13525		
	NCPPB 667	P. fluorescens bv. II[c]	ATCC 10844	Lactuca sativa	
	NCPPB 2644	P. fluorescens bv. II[d]	PDDCC 5708	Medicago sativa	
	NCPPB 1797	P. fluorescens bv. III			
	NCPPB 325	P. solanacearum	ATCC 11696	Lycopersicon esculentum	
	TCRI 01	P. solanacearum[e]		Nicotiana tabacum	Kroondal, S.A.[f]
	TCRI 02	P. solanacearum[e]		Lycopersicon esculentum	Kroondal, S.A.[f]
	VOPRI 5	P. solanacearum[e]		Solanum tuberosum	Clanwilliam, S.A.[g]
	VOPRI 26	P. solanacearum[e]		Solanum tuberosum	Makoppa, S.A.[g]
	NCPPB 2356	P. syringae pv. mellea	PDDCC 5711	Nicotiana tabacum	
	NCPPB 2995	P. syringae pv. morsprunorum	ATCC 19322	Prunus spp.	
	NCPPB 639	P. syringae pv. savastanoi	ATCC 13522	Olea europaea; Fraxinus spp.	
	NCPPB 281	P. syringae pv. syringae	ATCC 19310	Syringa vulgaris	
	NCPPB 1427	P. syringae pv. tabaci	PDDCC 2835	Nicotiana tabacum	
	NCPPB 2192	P. tolaasii		Cultivated mushrooms	
	NCPPB 635	P. viridiflava	ATCC 13223	Phaseolus vulgaris	
II	NCPPB 457	X. axonopodis	ATCC 19312	Axonopus spp.	
	NCPPB 528	X. campestris pv. campestris	PDDCC 13	Brassica spp.	
	NCPPB 101	X. campestris pv. cassavae	PDDCC 204	Manihot spp.	
	NCPPB 490	X. campestris pv. mangiferaeindicae	PDDCC 5740	Mangifera indica	
	0823	X. campestris pv. mangiferaeindicae		Mangifera indica	Nambour, Queensland[h]
	0836	X. campestris pv. mangiferaeindicae		Mangifera indica	Gin Gin, Queensland[h]
	UP 89	"P. mangiferaeindicae"[i]		Mangifera indica	Northern Transvaal, S.A.
	D42	X. campestris pv. mangiferaeindicae		Mangifera indica	Eastern Transvaal, S.A.
	NCPPB 1834	X. campestris pv. manihotis	PDDCC 5741	Manihot spp.	
	NCPPB 3035	X. campestris pv. phaseoli	PDDCC 5834	Phaseolus spp.	
	NCPPB 416	X. campestris pv. pruni	PDDCC 51	Prunus spp.	
	NCPPB 2475	X. campestris pv. viticola	PDDCC 3867	Vitis vinifera	
	NCPPB 1469	X. fragariae	PDDCC 5715	Fragaria vesca	
III	NCPPB 1683	P. hibiscicola	ATCC 19867		
	NCPPB 1974	X. maltophilia	ATCC 136237		
Not de-	NCPPB 2607	P. amygdali		Prunus dulcis	
signa-	NCPPB 1962	P. cepacia	ATCC 25416	Allium cepa	
ted	NCPPB 2217	Xy. ampelinus[j]	PDDCC 4298	Vitis vinifera	

Electrophoresis was performed in an anodic system at a constant current of 25 mA/gel for the stacking gel and 35 mA/gel for the separating gel. Running time was 5 to 6 h at 15 °C.

Gels were fixed and stained overnight according to Anderson's Brilliant Blue R staining procedure (*Anderson* and *Anderson*, 1977) using Coomassie Brilliant Blue R-250 in ethanol and 10% acetic acid. Destaining was done in four successive steps according to the same procedure, using 5% acetic acid and 95% ethanol.

Spectrophotometry and normalization of spectrophotometric tracings. Destained gels were scanned in a Beckman DU-8 spectrophotometer with a gel scan module adjusted to the following settings: absorption; wavelength 560 nm; slit width 1.0 mm; gel slit width 0.2 mm; chart speed 30.0 cm/min; gel speed 10.0 cm/min; The bottom centimetre of the gel was used as a blank.

Normalization of the spectrophotometric scans was performed by the method of *Kersters* and *De Ley* (1975). The best fit between each pair of traces was obtained by laterally shifting one trace with respect to the other in single point steps of approx. 5 points on either side of the initial alignment with respect to the reference proteins (*Owen* and *Jackman*, 1982).

Numerical analysis of electrophoregrams. The Pearson product-moment correlation coefficient, r, (*Sokal* and *Sneath*, 1963) between any pair of normalized spectrophotometric tracings of protein patterns was calculated (*Kersters* and *De Ley*, 1975). The resulting r-matrix was transformed to a distance matrix (*Rohlf* and *Sokal*, 1965) and clustered by the unweighted average pair-group method (*Zupan*, 1982). The distance values of all clustering levels were again transformed to r values. All calculations were performed using the IBM-mainframe computer of the Institute for Computer Sciences, University of Pretoria.

Photography and drying of gels. Gels were photographed on a light table with a Canon AE-1 camera fitted with a macro lens (28 mm focal length). Kodak Ektachrome 50 Tungsten film was used.

Gels were soaked in an aqueous solution of 1% glycerol −10% acetic acid for 2 d and then dried between two layers of cellophane (boiled beforehand in a 5% Na₂CO₃-50 mM EDTA solution) on a Bio-Rad Gel Slab Dryer (Model 224) and filed for later reference.

Results and Discussion

Morphological, physiological and biochemical features

Most of the results from the phenotypic tests correspond to those in the literature (*Bradbury*, 1984; *Palleroni*, 1984). Gram-stain negative, catalase production and motility were the only positive features for all 38 strains used. The following 5 features were negative throughout: production of indole; fermentative reaction using glucose in *Hugh* and *Leifson's* O/F medium and acid production from inulin, salicin and α-methyl-D-glucoside. The results of the tests giving differential reactions are summarised in Table 2. The average reproducibility of tests was 96%.

The problem of differentiating between pathovars by means of biochemical tests, is illustrated by the *P. syringae* and *X. campestris* groups for which, in most cases, all the features were either positive or negative respectively for all strains tested. This resulted in a > 90% S clustering level for these individual pathovar groups, indicating that they share 90% or more of their features, and therefore only a few phenotypic features were useful for differentiation.

Numerical analysis of phenotypic features

The results of the numerical analysis of the phenotypic features correspond to a high degree with the present taxonomic grouping in these genera. Three phenons and three solitary strains were differentiated at 75,7% S, clearly reflecting the three rRNA homology groups I, II and V as defined by *Palleroni* (1984).

Group 2 of phenon I corresponds to rRNA group I. Also assigned to this group of fluorescent pseudomonads are the two taxonomically unattached fluorescent strains *P. caricapapayae* and *P. tolaasii*. The unexpected and unlikely inclusion of *P. avenae* in this group can be ascribed to the similarity shown by *P. angulata* and *P. avenae*, which is probably accidental because of the amount and assembly of the phenotypic tests used.

All the *P. solanacearum* strains clustered in a separate group, corresponding to rRNA homology group II. *P. cepacia* was however not included in this group. For the *P. solanacearum* strains, no correlation was found between host-specificity and phenotypic features.

Except for *X. maltophilia*, the xanthomonads clustered in a single phenon, corresponding to rRNA group V. All the *X. campestris* pathovars grouped together, with *X. campestris* pv. *manihotis* (a nonpigmented pathovar) included in the core of the nonpigmented *X. campestris* pv. *mangiferaeindicae* group.

The high relatedness of *X. maltophilia* and *P. hibiscicola* (99% S) in a separate group supports the view that they might be synonyms (*Palleroni*, 1984). They did however not cluster as close to the *Xanthomonas* group as suggested by *Swings* et al. (1983) and *De Vos* et al. (1985). They were in fact less related to the *Xanthomonas* group than the majority of the *Pseudomonas* strains studied.

P. amygdali, a non-fluorescent pseudomonad of uncertain affinity (*Palleroni*, 1984), clustered totally separately.

Fourteen of the 17 different *Pseudomonas* and *Xanthomonas* species clustered above the specie level (80–85% S) (*Austin* and *Priest*, 1986). This can be ascribed to the limited number of tests (53) used in this study, but again illustrates the high phenotypic relatedness of species of these two genera.

The taxonomic structure of this group of strains as determined by numerical analysis of their electrophoretic protein patterns

Whereas the overall grouping obtained by using the phenotypic features and the similarity coefficient corresponds to the rRNA homology groups as suggested by *Palleroni* (1984), the overall grouping obtained by the numerical analysis of electrophoregrams (Figs. 2 and 3) corresponds more to the separate DNA homology groups suggested by the same author. However, an excellent agreement is found between the separate subgroupings obtained by numerical analysis of phenotypic features and of protein electrophoregrams.

Four electrophoretic clusters and one solitary strain were delineated at r = 0,64. *P. solanacearum* (NCPPB 325) and *P. avenae* each clustered separately at a lower level. Reproducibility limits of electrophoresis were above r = 0.95 as determined with three independently grown cultures of the same strain.

Table 2. Differential phenotypic features
a) *P. marginalis* pv. *alfalfae*.
b) *P. marginalis* pv. *marginalis*.

	GROUP 1 (5 strains)						GROUP 2 — SUBGROUP 1 (6 strains)							GROUP 2 — SUBGROUP 2 (5 strains)						GROUP 2 — SUBGROUP 3 (2 strains)			2 strains			
	P. solanacearum (NCPPB 325)	*P. solanacearum* (TCRI 01)	*P. solanacearum* (TCRI 02)	*P. solanacearum* (VOPRI 5)	*P. solanacearum* (VOPRI 26)	% of strains positive	*P. syringae* pv. *tabaci*	*P. syringae* pv. *syringae*	*P. syringae* pv. *morsprunorum*	*P. syringae* pv. *mellea*	*P. syringae* pv. *savastanoi*	*P. caricapapayae*	% of strains positive	*P. fluorescens* bv. I	*P. fluorescens* bv. II[a]	*P. fluorescens* bv. II[b]	*P. fluorescens* bv. III	*P. tolaasii*	% of strains positive	*P. angulata*	*P. avenae*	No. of strains positive	*P. viridiflava*	*P. aeruginosa*	*P. cepacia*	*P. amygdali*
Production of:																										
Fluorescent pigments	-	-	-	-	-	0	+	+	+	+	+	+	100	+	+	+	+	+	100	+	-	1	+	+	-	-
Xanthomonadins	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-
Urease activity	-	-	-	-	-	0	+	+	+	+	+	+	100	+	+	-	+	+	80	-	-	0	-	-	-	-
Oxidase reaction	+·	+	+	+	+	100	-	-	-	-	-	-	0	+	+	+	+	+	100	-	-	0	-	+	+	+
H₂S from cysteine	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	-	+	+	80	+	+	2	+	+	-	-
Asparagine as sole source of C and N	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	+
Protein digestion:																										
"purple milk"	+	-	+	-	-	40	-	+	-	-	-	+	33	-	+	+	+	+	80	+	+	2	+	+	+	+
Milk agar plates	-	-	-	-	-	0	-	-	-	-	-	-	0	-	+	+	+	+	80	-	-	0	-	-	+	+
Nitrate reduction	+	+	+	+	+	100	-	-	-	-	-	-	0	-	+	-	-	-	20	-	+	1	-	-	-	-
Acetoin production	-	-	-	-	-	0	-	+	+	+	-	+	67	-	-	-	-	-	0	-	-	0	-	-	-	-
Oxidative (H & L)	-	+	+	+	+	80	+	+	+	+	+	+	100	+	+	+	+	-	100	+	+	2	+	+	+	-
Arginine dihydrolase	-	-	-	-	-	0	-	-	-	-	-	+	17	+	+	+	+	+	100	+	+	2	+	+	-	-
β-Galactosidase	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-
PHB accumulation	+	+	+	+	+	100	-	-	-	-	-	-	0	-	-	-	-	-	0	-	+	1	-	-	+	-
Hydrolysis of:																										
Gelatin	-	-	-	-	-	0	+	+	+	-	-	+	67	+	+	+	+	+	100	+	+	2	+	+	+	+
Starch	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	+	-
Aesculin	+	-	+	-	-	40	+	+	+	+	+	+	100	+	+	+	+	-	80	-	-	0	+	-	+	-
Tween 80 (esterase)	+	+	+	+	+	100	+	-	+	+	+	+	83	+	+	-	+	+	80	+	+	2	+	+	+	-
Tyrosinase	+	+	+	+	+	100	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-
Acid production on Dye's medium C from:																										
L(+)-arabinose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	+
Cellobiose	-	+	+	+	+	80	-	-	-	-	-	-	0	-	+	-	+	+	60	+	+	2	-	-	-	-
Fructose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	+
Galactose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	-
Glucose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	-
Mannose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	-	+	-
Raffinose	-	-	-	-	-	0	+	+	+	-	-	+	67	+	+	+	-	-	60	+	-	1	-	-	+	-
Melibiose	-	-	-	-	-	0	+	+	+	+	+	+	100	+	+	+	+	+	100	+	-	1	+	+	-	+
Melezitose	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	+
Rhamnose	-	-	-	-	-	0	+	+	+	+	-		83	+	+	+	+	+	100	-	-	0	-	-	-	+
Trehalose	+	+	+	+	+	100	-	-	-	-	-	-	0	+	+	+	+	+	100	+	-	1	+	+	+	+
Xylose	+	+	+	+	+	100	+	+	+	+	-	+	83	+	+	+	+	+	100	+	+	2	+	+	+	+
Sucrose	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	-	-	60	-	-	0	-	-	+	-
Lactose	-	+	+	+	+	80	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	+	-
Maltose	-	+	+	+	+	80	-	-	-	-	-	-	0	-	+	+	-	+	60	-	+	1	-	-	+	-
Ribose	+	+	+	-	-	60	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	-
meso-Inositol	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	-	1	+	-	+	-
Adonitol	-	-	-	-	-	0	-	-	+	-	-·		17	+	+	-	+	+	80	-	-	0	-	-	+	-
Dulcitol	-	+	-	-	-	20	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	+	-
Mannitol	-	-	-	-	-	0	+	+	+	+	+	+	100	+	-	-	+	-	40	+	-	1	+	+	+	-
Sorbitol	-	-	-	-	-	0	+	+	+	+	+	+	100	+	+	+	-	+	80	+	+	2	+	+	+	+
Erythritol	-	-	-	-	-	0	+	+	+	+	-	+	83	+	+	+	+	+	100	+	+	2	+	-	-	-
Dextrin	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-
Starch	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	+
Glycerol	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	+	+	+	100	+	+	2	+	+	+	-
Glycogen	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	0	-	-	-	-

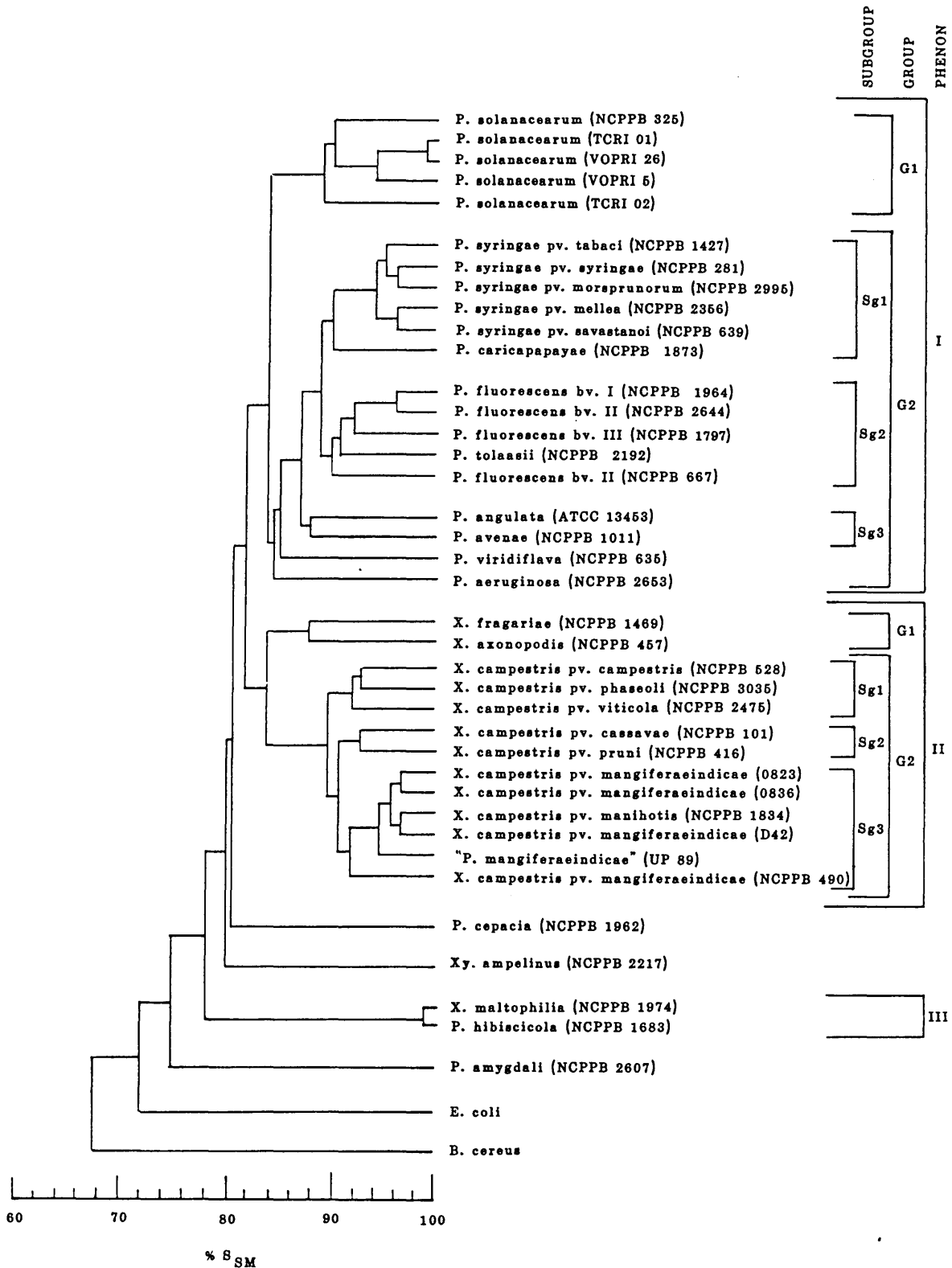|	PHENON II																		PHENON III			
	GROUP I			GROUP 2																		
				SUB-GROUP I	SUB-GROUP 2					SUBGROUP 3												
	2 strains			5 strains						6 strains								2 strains			
	X. fragariae	*X. axonopodis*	No. of strains positive	*X. campestris pv. campestris*	*X. campestris pv. phaseoli*	*X. campestris pv. viticola*	*X. campestris pv. cassavae*	*X. campestris pv. pruni*	% of strains positive	*X. campestris pv. manihotis*	*X. c. pv. mangiferaeindicae (NCPPH)*	*X. c. pv. mangiferaeindicae (0823)*	*X. c. pv. mangiferaeindicae (0836)*	*P. mangiferaeindicae (UP 89)*	*X. c. pv. mangiferaeindicae (D42)*	% of strains positive	*X. maltophilia*	*P. hibiscicola*	No. of strains positive	*Xy. ampelinus*
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Production of:																				
Fluorescent pigments	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Xanthomonadins	+	+	2	+	+	−	+	+	80	−	−	−	−	−	−	0	−	−	0	−
Urease activity	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	+
Oxidase reaction	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
H₂S from cysteine	−	+	1	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	+
Asparagine as sole source of C and N	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	+	+	2	−
Protein digestion:																				
"purple milk"	−	+	1	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	−
Milk agar plates	−	−	0	+	.+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	−
Nitrate reduction	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	+	+	2	−
Acetoin production	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Oxidative (H & L)	+	−	1	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	+
Arginine dihydrolase	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
β-Galactosidase	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	+
PHB accumulation	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Hydrolysis of:																				
Gelatin	+	−	1	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	−
Starch	+	+	2	+	+	+	−	−	80	+	−	−	−	−	−	13	−	−	0	−
Aesculin	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	−
Tween 80 (esterase)	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	+	+	2	+
Tyrosinase	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	+	1	−
Acid production on Dye's medium C from:																				
L(+)-arabinose	−	−	0	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	+
Cellobiose	−	−	0	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	−
Fructose	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	+
Galactose	+	−	1	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	+
Glucose	+	+	2	+	+	+	+	+	100	+	−	+	+	+	+	88	+	+	2	−
Mannose	+	−	1	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	−
Raffinose	−	−	0	−	+	+	+	−	60	−	+	+	+	+	+	88	−	−	0	−
Melibiose	−	−	0	+	+	+	−	−	60	+	+	+	+	+	+	100	−	−	0	−
Melezitose	−	−	0	−	−	−	−	−	0	−	+	−	−	+	−	38	−	−	0	−
Rhamnose	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Trehalose	+	+	2	+	+	+	−	+	80	+	+	+	+	+	+	100	−	−	0	−
Xylose	−	−	0	−	−	−	+	+	40	−	−	+	+	+	−	50	−	−	0	−
Sucrose	+	+	2	+	+	+	+	+	100	+	+	+	+	+	+	100	−	−	0	−
Lactose	−	−	0	−	−	−	+	+	40	−	−	−	−	−	−	0	−	−	0	−
Maltose	−	−	0	+	+	+	+	−	80	−	−	−	−	−	−	0	−	−	0	−
Ribose	−	−	0	+	+	+	−	−	60	−	+	+	−	+	+	67	−	−	0	−
meso-Inositol	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Adonitol	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Dulcitol	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Mannitol	−	−	0	+	−	+	−	−	40	−	−	−	−	−	−	0	−	−	0	−
Sorbitol	−	−	0	−	−	−	+	−	20	−	−	−	−	−	−	0	−	−	0	−
Erythritol	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Dextrin	−	−	0	+	+	+	+	−	80	−	−	−	+	−	−	13	+	−	1	+
Starch	−	−	0	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	0	−
Glycerol	−	−	0	+	+	+	−	+	80	+	+	+	+	+	+	100	−	−	0	+
Glycogen	−	−	0	+	+	−	−	−	40	−	−	−	−	−	−	0	−	−	0	−

Fig. 1. Phenogram of the similarity coefficient, S_{SM}, grouped by the unweighted average linkage cluster analysis, showing the taxonomic relationships between 24 phytopathogenic *Pseudomonas* and 14 *Xanthomonas* spp. and pathovars based on phenotypic features.
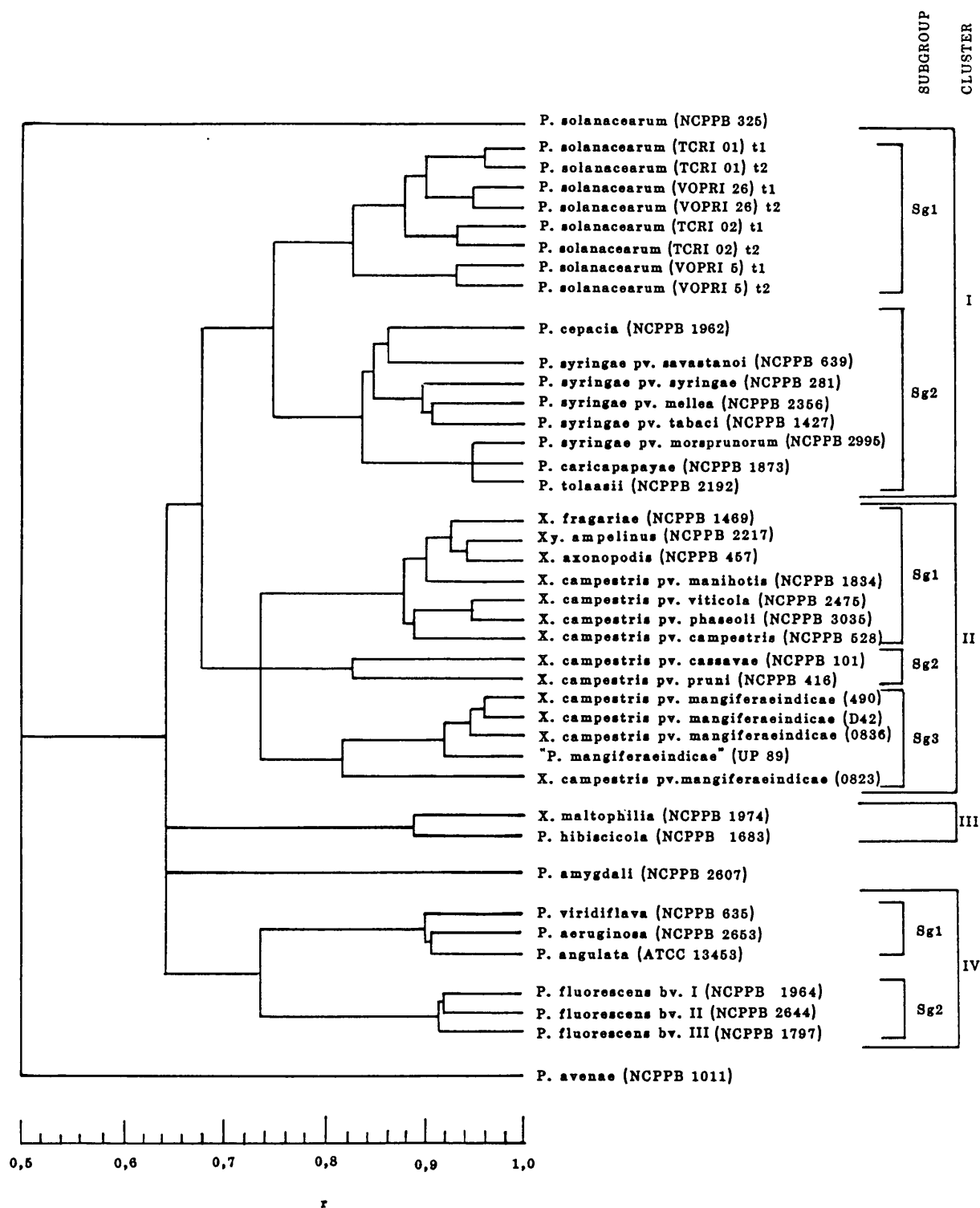
SUBGROUP

CLUSTER

P. solanacearum (NCPPB 325)

P. solanacearum (TCRI 01) t1
P. solanacearum (TCRI 01) t2
P. solanacearum (VOPRI 26) t1
P. solanacearum (VOPRI 26) t2
P. solanacearum (TCRI 02) t1
P. solanacearum (TCRI 02) t2
P. solanacearum (VOPRI 5) t1
P. solanacearum (VOPRI 5) t2

Sg1

P. cepacia (NCPPB 1962)

P. syringae pv. savastanoi (NCPPB 639)
P. syringae pv. syringae (NCPPB 281)
P. syringae pv. mellea (NCPPB 2356)
P. syringae pv. tabaci (NCPPB 1427)
P. syringae pv. morsprunorum (NCPPB 2995)
P. caricapapayae (NCPPB 1873)
P. tolaasii (NCPPB 2192)

Sg2

I

X. fragariae (NCPPB 1469)
Xy. ampelinus (NCPPB 2217)
X. axonopodis (NCPPB 457)
X. campestris pv. manihotis (NCPPB 1834)
X. campestris pv. viticola (NCPPB 2475)
X. campestris pv. phaseoli (NCPPB 3035)
X. campestris pv. campestris (NCPPB 528)

Sg1

X. campestris pv. cassavae (NCPPB 101)
X. campestris pv. pruni (NCPPB 416)

Sg2

X. campestris pv. mangiferaeindicae (490)
X. campestris pv. mangiferaeindicae (D42)
X. campestris pv. mangiferaeindicae (0836)
"P. mangiferaeindicae" (UP 89)
X. campestris pv. mangiferaeindicae (0823)

Sg3

II

X. maltophilia (NCPPB 1974)
P. hibiscicola (NCPPB 1683)

III

P. amygdali (NCPPB 2607)

P. viridiflava (NCPPB 635)
P. aeruginosa (NCPPB 2653)
P. angulata (ATCC 13453)

Sg1

P. fluorescens bv. I (NCPPB 1964)
P. fluorescens bv. II (NCPPB 2644)
P. fluorescens bv. III (NCPPB 1797)

Sg2

IV

P. avenae (NCPPB 1011)

0,5   0,6   0,7   0,8   0,9   1,0

r

Fig. 2. Dendrogram of the correlation coefficient, r, grouped by the unweighted average linkage cluster analysis, showing the axonomic relationships between 23 phytopathogenic *Pseudomonas* and 14 *Xanthomonas* spp. and pathovars based on protein lectrophoretic patterns.

Fig. 3. Dendrogram showing protein electrophoregrams of the 37 phytopathogenic *Pseudomonas* and *Xanthomonas* species and pathovars.

The separate clustering of the reference strain of *P. solanacearum* (NCPPB 325) is in accordance with results that this branch should be removed from the genus *Pseudomonas* (*De Vos* et al., 1985). Despite phenotypic simmilarities, the local strains of this species did not cluster with the reference strain, accentuating the wide strain variation of this pathogen (*Garrett*, 1982). For the local *P. solanacearum* strains a very high correlation (r > 0.92) was found between the two different colony types (virulent and avirulent) from each strain. Again no correlation was found between host of isolation and electrophoretic clustering or virulence and electrophoretic clustering.

P. caricapapayae and *P. tolaasii* were satisfactorily placed in the *P. syringae* group. The tobacco pathogens, *P. syringae* pv. *mellea* and *P. syringae* pv. *tabaci*, clustered at r = 0.91, indicating a possible correlation between host-specificity and protein profiles in this group.

Of importance is the electrophoretic differentiation of members of the *P. fluorescens* rRNA branch on the basis of their phytopathogenic abilities. This is illustrated by the separate clustering of the *P. syringae* subgroup (Cluster I – Sg2) and the *P. fluorescens* cluster (Cluster IV), the latter consisting of saprophytic species or species not yet fully specialised as phytopathogens (*Palleroni*, 1984).

The xanthomonads clustered as a homogeneous group, clearly differentiated from the pseudomonads. All the *X. campestris* pv. *mangiferaeindicae* strains clustered in a single subgroup. The reclassification of "*P. mangiferaeindicae*" (UP 89) as *X. campestris* pv. *mangiferaeindicae* (*Robbs* et al., 1974) was again proven justified by this study. *Xy. ampelinus* was also included in the *Xanthomonas* group. As previously explained, the culture used for electrophoresis did not correspond to the accepted description of this species, despite the fact that it was obtained from three separately procured pure cultures. The culture used for electrophoresis might therefore not have been representative of the species.

X. maltophilia and *P. hibiscicola* formed a separate cluster, proving a close relationship between them. Again, as in the phenogram, the *X. maltophilia* group did not cluster significantly close to the *Xanthomonas* group and joined the rest of the strains only at r = 0.64. Supported by our electrophoretic (and classical phenotypic) results, we find *Palleroni's* (1984) "more rational" suggestion that a new genus should be created for the former *P. maltophilia*, which, together with *Xanthomonas*, could constitute a separate new taxon, more acceptable than the inclusion of "*P.*" *maltophilia* in the genus *Xanthomonas* (*Swings* et al., 1983). In view of the significant differences between these two taxons, the latter reclassification is questionable (*Palleroni*, 1986), especially when it is considered that similarity maps of DNA-rRNA hybrids (*De Vos* and *De Ley*, 1983) and 16S rRNA cataloging (*Woese*, 1984) always placed "*P.*" *maltophilia* close to *Xanthomonas*, but never actually included it in this genus. DNA homologies between *X. maltophilia* strains and *Xanthomonas* were also found to be low (*Swings* et al., 1983).

As with the phenotypic features, *P. amygdali* clustered as a solitary species, justifying a more extensive study of this atypical species.

In cluster IV the *P. fluorescens* biovars formed a tight group, while the rest of the cluster consisted of the other fluorescent pseudomonads, namely *P. aeruginosa*, *P. viridiflava* and *P. angulata*. Although *P. angulata* was omitted from the Approved Lists of Bacterial Names (*Skerman* et al., 1980), it is still recognized as the causal organism of angular leaf spot of tobacco which is of worldwide distribution and which can range from a minor problem to the cause of a severe epidemic (*Akehurst*, 1981). *P. angulata* is furthermore regarded as identical with or closely related to *P. syringae* pv. *tabaci* because of the similarity in their disease symptoms (*Stapp*, 1930; *Hildebrand*, 1971; *Akehurst*, 1981). In the present study however, neither the phenotypic features nor the electrophoretic data provided any proof of this alleged relationship between them. In both our approaches *P. angulata* was found to be closely related to *P. aeruginosa*, which, interestingly enough, is also associated with leaf spot of tobacco (*Palleroni*, 1984). A thorough comparative study of the phenotypic properties and pathological differences of the strains concerned is necessary to resolve the uncertainty surrounding the exact identity of *P. angulata* and to establish whether the omission of *P. angulata* from the Approved Lists of Bacterial Names was justified.

P. avenae joined the rest of the strains at the low correlation level of r = 0.50, indicating that *P. avenae* should be removed from the genus *Pseudomonas*.

Our study shows that SDS- PAGE of the total soluble proteins of the bacterial cell is a reliable and objective method for the differentiation of phytopathogenic *Pseudomonas* and *Xanthomonas* spp.. The results of this method correlate to a great extent with those obtained from classical phenotypic features, as well as DNA homology studies and DNA-rRNA hibridization studies. It furthermore provides a practical method for the rapid differentiation of pathovars of these two genera that were previously differentiated primarily on the basis of their hosts of isolation.

Based on our electrophoretic results the following taxonomic proposals are made:

i) *P. caricapapayae* and *P. tolaasii* should be included in the *P. syringae* group as pathovars of *P. syringae*.

ii) A new genus should be created for *X. maltophilia* (*Van Zyl* and *Steyn*, manuscript in preparation).

iii) *P. avenae* should be generically reclassified.

iv) *P. amygdali* is an atypical pseudomonad and its taxonomical position should be reconsidered.

v) *P. angulata* is probably a valid nomenspecies. Our results indicate a probable reclassification of this strain as a pathovar of *P. aeruginosa*.

The aim of this study was not to solve all the problems in these two genera, but to accentuate the applicability of PAGE, as a taxonomic tool, in everyday phytobacterial practices, as it has been underestimated and neglected in this field for too long. Most of the taxonomical proposals are therefore mere suggestions which have to be followed up by more extensive study before definite taxonomic conclusions can be drawn.

for supplying the computer program for numerical analysis of electrophoregrams and Mr *J. I. Maritz* for the modification and implementation thereof. The assistance of Prof. *K. Kersters* and Dr. *J. J. Bezidenhout* in the implementation of the computer programs for cluster analysis, is gratefully acknowledged.

References

Akehurst, B. C.: Tobacco (*D. Rhind, G. Wrighley,* eds.) 2nd ed. London, Longman 1981

Anderson, N. G., Anderson, N. L.: High resolution two-dimensional electrophoresis of human plasma proteins. Proc. Nat. Acad. Sci. USA 74, 5421 (1977)

Austin, B., Priest, F. G.: Modern bacterial taxonomy. Workingham, Van Nostrand Reinhold 1986

Bradbury, J. F.: Genus II. Xanthomonas, pp. 199–210. In: Bergey's manual of systematic bacteriology, vol. 1 (*N. R. Krieg, J. G. Holt,* eds.), 9th ed. Baltimore, Williams and Wilkins 1984

Buddenhagen, I., Kelman, A.: Biological and physiological aspects of bacterial wilt caused by Pseudomonas solanacearum. Ann. Rev. Phytopath. 2, 203–230 (1964)

De Vos, P., De Ley, J.: Intra- and intergeneric similarities of Pseudomonas and Xanthomonas ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 33, 487–509 (1983)

De Vos, P., Goor, M., Gilis, M., De Ley, J.: Ribosomal ribonucleic acid cistron similarities of phytopathogenic Pseudomonas species. Int. J. system. Bact. 35, 169–184 (1985)

Dowson, W. J.: On the systematic position and generic names of the gram negative bacterial plant pathogens. Zbl. Bakt., II. Abt. 100, 177–193 (1939)

Dye, D. W.: The inadequacy of the usual determinative tests for the identification of Xanthomonas spp.. N.Z. J. Sci. 5, 393–416 (1962)

Dye, D. W.: Xanthomonas, pp. 45–49. In: Laboratory guide for identification of plant pathogenic bacteria (*N. W. Schaad,* ed.). Minnesota, Amer. Phytopath. Soc. 1980

El-Sharkawy, T. A., Huisingh, D.: Differentiation among Xanthomonas species by polyacrylamide gel electrophoresis of soluble proteins. J. gen. Microbiol. 68, 155–165 (1971)

Garrett, C. M. E.: Bacterial diseases of food plants – an overview, pp. 115–132. In: Bacteria and plants (*M. E. Rhodes-Roberts, F. A. Skinner,* eds.). London, Academic Press 1982

Hames, B. D.: An introduction to polyacrylamide gel electrophoresis, pp. 1–91. In: Gel electrophoresis of proteins (*B. D. Hames, D. Rickwood,* eds.). Oxford, IRL Press 1981

Harrigan, W. F., McCance, M. E.: Laboratory methods in microbiology. London, Academic Press 1966

Hayward, A. C.: Occurence of glycoside hydrolases in plant pathogenic and related bacteria. J. appl. Bact. 42, 407–411 (1977)

Hildebrand, D. C.: Pectate and pectin gels for differentiation of Pseudomonas spp. and other bacterial plant pathogens. Phytopathology 61, 1430–1436 (1971)

Hugh, R., Leifson, E.: The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. J. Bact. 66, 24–26 (1953)

Jackman, P. J. H.: Classification of Corynebacterium species from axillary skin by numerical analysis of electrophoretic protein patterns. J. med. Microbiol. 15, 485–492 (1982)

Kersters, K., De Ley, J.: Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. J. gen. Microbiol. 87, 333–342 (1975)

Kersters, K., De Ley, J.: Classification and identification of bacteria by electrophoresis of their proteins, pp. 273–297. In:

Microbiological classification and identification (*M. Goodfellow, R. G. Board,* eds.). London, Academic Press 1980

Kovacs, N.: Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature 178, 703 (1956)

Krieg, N. R., Jones, D.: Bacterial classification V. Serology and chemotaxonomy, pp. 979–982. In: Bergey's manual of systematic bacteriology (*P. H. A. Sneath, N. S. Mair, M. E. Sharpe, J. G. Holt,* eds.), 9th ed., vol. III. London, Williams and Wilkins 1986

Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685 (1970)

Migula, W.: Ueber ein neues System der Bakterien. Arb. Bakt. Inst. Techn. Hochsch. Karlsruhe 1, 235–238 (1894) (as quoted by *Palleroni,* 1984)

Moore, W. E. C., Hash, D. E., Holdeman, L. V., Cato, E. P.: Polyacrylamide slab gel electrophoresis of soluble proteins for studies of bacterial floras. Appl. Environ. Microbiol. 39, 900–907 (1980)

Owen, R. J., Jackman, P. J. H.: The simmilarities between Pseudomonas paucimobilis and allied bacteria derived from analysis of deoxyribonucleic acids and electrophoretic protein patterns. J. gen. Microbiol. 128, 2945–2954 (1982)

Oyaizu, H., Komagata, K.: Grouping of Pseudomonas species on the basis of cellular fatty acid compositions and the quinone system with special reference to the existence of 3-hydroxy fatty acids. J. gen. appl. Microbiol. 29, 17–40 (1983)

Palleroni, N. J.: Introduction to the family Pseudomonadaceae, pp. 655–665. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (*M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, H. G. Schlelgel,* eds.). Berlin, Springer-Verlag 1981

Palleroni, N. J.: Family I. Pseudomonadaceae, pp. 141–199. In: Bergey's manual of systematic bacteriology (*N. R. Krieg, J. G. Holt,* eds.), 9th ed., vol. I. Baltimore, Williams and Wilkins 1984

Palleroni, N. J.: Taxonomy of the pseudomonads, pp. 3–25. In: The bacteria: the biology of Pseudomonas (*J. R. Sokatch,* ed.). London, Academic Press 1986

Palmer, B. C., Cameron, H. R.: Comparison of plantpathogenic pseudomonads by disc-gel electrophoresis. Phytopathology 61, 984–986 (1971)

Robbs, C. F., Ribeiro, R. de L. D., Kimura, O.: Sobre a posicao taxonomica de Pseudomonas mangiferaeindicae Patel et al. 1948, agente causal da "mancha bacteriana" das folhas da mangueira (*Mangifera indica* L.). Arg. Univ. Fed. Rural Rio de Janeiro 4, 11–14 (1974)

Rohlf, F. J., Sokal, R. R.: Coefficients of correlation and distance in numerical taxonomy. Univ. Kansas Sci. Bull. 45, 3–27 (1965)

Sands, D. C., Schroth, M. N., Hildebrand, D. C.: Pseudomonas, pp. 36–44. In: Laboratory guide for identification of plant pathogenic bacteria (*M. W. Schaad,* ed.). Minnesota, Amer. Phytopath. Soc. 1980

Schroth, M. N., Hildebrand, D. C., Starr, M. P.: Phytopathogenic members of the genus Pseudomonas, pp. 701–718. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (*M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, H. G. Schlegel,* eds.). Berlin, Springer-Verlag 1981

Sierra, G.: A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. Antonie v. Leeuwenhoek 23, 15 (1957)

Skerman, V. B. D., McGowan, V., Sneath, P. H. A.: Approved lists of bacterial names. Int. J. system. Bact. 30, 225–420 (1980)

neath, P. H. A., Sokal, R. R.: Numerical taxonomy. The principles and practice of numerical classification. San Francisco, W. H. Freeman and Co. 1973

okal, R. R., Michener, C. D.: A statistical method for evaluating systematic relationships. Univ. Kansas Sci. Bull. *38*, 1409–1438 (1958)

okal, R. R., Sneath, P. H. A.: Principles of numerical taxonomy. San Francisco, W. H. Freeman and Co. 1963

tapp, C.: Bakterielle Tabakkrankheiten und ihre Erreger. Angew. Bot. *12*, 241–274 (1930) (as quoted by *Braun*, 1937)

tarr, M. P.: The genus *Xanthomonas*, pp. 742–763. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (*M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, H. G. Schlegel*, eds.). Berlin, Springer-Verlag 1981

tegemann, H., Burgermeister, W., Francksen, H., Krögerrecklenfort, E.: Panta-manual on gel electrophoresis and isoelectric focusing. Braunschweig, Institut für Biochemie, 1987

teyn, P. L., Viljoen, N. M., Kotzé, J. M.: The causal organism of bacterial black spot of mangoes. Phytopathology *64*, 1400–1404 (1974)

tolp, H., Starr, M. P., Baigent, N. L.: Problems in speciation of phytopathogenic pseudomonads and xanthomonads. Ann. Rev. Phytopath. *3*, 231–264 (1965)

wings, J., De Vos, P., Van den Mooter, M., De Ley, J.: Transfer of *Pseudomonas maltophilia* Hugh 1981 to the genus *Xanthomonas* as *Xanthomonas maltophilia* (Hugh 1981) comb. nov.. Int. J. system. Bact. *33*, 409–413 (1983)

Thornley, M.: The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. J. appl. Bact. *23*, 37 (1960)

Van den Mooter, M., Steenackers, M., Maertens, C., Gosselé, F., De Vos, P., Swings, J., Kersters, K., De Ley, J.: Differentiation between *Xanthomonas campestris* pv. *graminis* (ISPP List 1980), pv. *phleipratensis* (ISPP LIst 1980) emend., pv. *poae* Egli and Schmidt 1982 and pv. *arrhenatheri* Egli and Schmidt 1982, by numerical analysis of phenotypic features and protein gel electrophoregrams. J. Phytopath. *118*, 135–156 (1987)

Van Zyl, E., Kotzé, J. M., Steyn, P. L.: Isolation of *Xanthomonas campestris* pv. *mangiferaeindicae* from gall fly-induced lesions on mango leaves. Phytophylactica *20*, 89–90 (1988)

Vera Cruz, C. M., Gosselé, F., Kersters, K., Segers, P., Van den Mooter, M., Swings, J., De Ley, J.: Differentiation between *Xanthomonas campestris* pv. *oryzae*, *Xanthomonas campestris* pv. *oryzicola* and the bacterial "brown blotch" pathogen on rice by numerical analysis of phenotypic features and protein gel electrophoregrams. J. gen. Microbiol. *130*, 2983–2999 (1984)

Willems, A., Gillis, M., Kersters, K., Van den Broecke, L., De Ley, J.: Transfer of *Xanthomonas ampelina* Panagopoulos 1969 to a new genus, *Xylophilus* gen. nov., as *Xylophilus ampelinus* (Panagopoulos 1969) comb. nov.. Int. J. system. Bact. *37*, 422–430 (1987)

Woese, C. R., Blanz, P., Hahn, C. M.: What isn't a pseudomonad: the importance of nomenclature in bacterial classification. System. Appl. Microbiol. *5*, 179–195 (1984)

Zupan, J.: Clustering of large data sets (*D. Bawden*, ed.). Chichester, Research Studies Press 1982

Elritha van Zyl, Dept. of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, Republic of South Africa

Taxonomy of the Phytopathogenic <u>Pseudomonas</u> Species Belonging to
the Acidovorans rRNA Complex

ELRITHA VAN ZYL   and   P.L. STEYN

Department of Microbiology and Plant Pathology,
University of Pretoria, Pretoria 0002,
Republic of South Africa

SUMMARY

The taxonomic relationships of representatives of the majority of the phytopathogenic Pseudomonas species belonging to the acidovorans rRNA complex were determined by numerical analysis of polyacrylamide gel electrophoregrams of soluble proteins. Two well defined clusters were formed, with a very low correlation between them. "[P.] setariae" was found not to be synonymous with [P.] avenae, but probably represents a different species closely related to [P.] rubrilineans. [P.] avenae (NCPPB 2402) is probably misnamed.

INTRODUCTION

The elucidation of relationships among bacteria at the generic and suprageneric levels is one of the main problems to be solved in modern bacterial taxonomy (DE VOS and DE LEY, 1983). For this reason much attention has been devoted recently to deoxyribo-nucleic acid - ribosomal ribonucleic acid (DNA - rRNA) hybridization and rRNA sequence studies. However, the elucidation of relationships at the subgeneric level is equally important for the classification and identification of strains. Most of the recent taxonomic studies of Pseudomonas species have been concerned with nucleic acid analysis (DE VOS and DE LEY, 1983; DE VOS et al., 1985; DE VOS et al., 1989). Very few studies have been carried out to revise and improve the phenotypic definitions of species and to determine their generic positions and subgeneric relationships (GAVINI et al., 1989).

Presently, Section III of Pseudomonas (the [Pseudomonas] acidovorans rRNA branch) in Bergey's manual of systematic Bacteriology (PALLERONI, 1984) contains no phytopathogens. Recent DNA-rRNA hybridization studies, however, have assigned the following phytopathogens to this rRNA branch (since all of these Pseudomonas spp. are considered to be generically misnamed, this is indicated by brackets): [Pseudomonas] avenae (syn. "[P.] alboprecipitans"), [Pseudomonas] cattleyae, [Pseudomonas]

pseudoalcaligenes subsp. citrulli, [Pseudomonas] pseudoalcaligenes subsp. konjaci, [Pseudomonas] rubrilineans and "[Pseudomonas] setariae" (BYNG et al., 1980; DE VOS et al., 1985; GOOR et al., 1986; WILLEMS et al., 1987). As the results of DNA-rRNA hybridization studies do not generally allow species separation within a rRNA branch (DE VOS et al., 1985), knowledge of the natural relationships between these species is incomplete.

In a previous study of phytopathogenic Pseudomonas and Xanthomonas species (VAN ZYL and STEYN, 1990), we showed that numerical analysis of electrophoretic patterns of the total soluble proteins of the bacterial cell is a reliable tool for establishing taxonomic relationships among phytopathogenic pseudomonads and xanthomonads from the level of pathovar to genus. The results of our study correlated well with those obtained from classical phenotypic features, DNA homology and DNA-rRNA hybridization studies.

The present study, an extension of our previous work (VAN ZYL and STEYN, 1990), deals with the taxonomic relationships of the majority of the phytopathogenic species of the acidovorans rRNA branch as determined by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) of soluble proteins.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The strains used are listed in Table 1. The bacteria were grown at 28°C on GYCA medium (VERA CRUZ et al., 1984) and maintained on GYCA slants at room temperature. For electropho-retic studies cultures were incubated with shaking at 28°C for 72 h in 10 ml Standard 1 nutrient broth (Merck) supplemented with 0,15% K_2HPO_4 and 0,15% $Mg_2SO_4 . 7H_2O$.

Preparation of whole cell extracts for electrophoresis

Whole cell protein extracts were prepared as described by VAN ZYL and STEYN (1990), with the following modification: 0,01 g (wet weight) of harvested cells from each strain was resuspended in 1 ml sample buffer (LAEMMLI, 1970) in an Eppendorf centrifuge tube and sonicated for 30 s at a 40 watt output with a Cole-Parmer ultrasonic processor (series 4710) fitted with a 3 mm microtip.

Electrophoresis, densitometry and numerical analysis of protein patterns

One-dimensional vertical electrophoresis, using the discontinuous buffer system of LAEMMLI (1970), was performed as previously described (VAN ZYL and STEYN, 1990). Destained gels were scanned with a Hoefer GS 300 scanning densitometer connected to a Lloyd Graphic 1000 Y/t strip chart recorder. The wavelength used was 580 nm and the chart speed 5 mm/s. The densitometric scans were normalized by the method of KERSTERS and DE LEY (1975). Computer-assisted numerical analysis of normalized densitometric tracings was done by the procedure of VAN ZYL and STEYN (1990).

RESULTS AND DISCUSSION

The dendrogram obtained after numerical analysis of the data is shown in Fig 1. Two well defined clusters were formed. Cluster I contained Comamonas acidovorans, [P.] pseudoalcaligenes subsp. citrulli, [P.] rubrilineans and "[P.] setariae"; cluster II all the [P.] avenae strains.

Although located in cluster I, C. acidovorans, the only non-phytopathogenic species included in this study, was only distantly related to the phytopathogens of this cluster (r = 0,4).

Our results are in partial agreement wiht those of WILLEMS et al. (1987). These authors proposed an acidovorans rRNA subbranch containing C. acidovorans, with [P.] pseudoalcaligenes subsp. citrulli, [P.] rubrilineans and "[P.] setariae" located on the Tm(e) dendrogram at the branching point of the acidovorans subbranch. [P.] avenae, on the other hand, was located close to the [Alcaligenes] paradoxus rRNA subbranch, thus substantially removed from the other phytopathogens (WILLEMS et al., 1987). On the basis of cellular fatty acid composition and the quinone system, OYAIZU and KOMAGATA (1983) also placed C. acidovorans and [P.] avenae in separate groups. We therefore cannot agree with WILLEMS et al. (1989) that [P.] avenae is closely related to [P.] rubrilineans and "[P.] setariae". The [P.] rubrilineans and "[P.] setariae" strains used by us were the same as those used by WILLEMS et al. (1989) but the [P.] avenae strains were different.

The low correlation between the two clusters in Fig 1 (r = 0,35) supports the postulation that each subbranch in the acidovorans rRNA complex deserves generic rank and that many species of this group are therefore generically misnamed (WILLEMS et al., 1987). The low correlation between species within a cluster also indicates that the organisms of the acidovorans rRNA complex show great diversity (WILLEMS et al., 1987; DE VOS et al., 1989).

The taxonomic status of "[Pseudomonas] setariae" is not clear. The species name was not retained on the Approved Lists of Bacterial Names (SKERMAN et al., 1980; MOORE et al., 1985), nor is it on Validation Lists 17 to 29 (International Journal of Systematic Bacteriology, 1985-1989). In the catalogue of the National Collection of Plant Pathogenic Bacteria (1988) "[P.] setariae" (NCPPB 1392) is listed as a synonym of [P.] avenae. However, it is noted that this culture has not been checked by the NCPPB nor by a recognised authority and that its authenticity is doubtful. According to our results "[P.] setariae" (NCPPB 1392) is not a synonym of [P.] avenae, but a different and separate species. We are therefore tempted to suggest that the

name "[P.] setariae" should be revived as cultures are available and the name is in general use (DE VOS et al., 1985; WILLEMS et al., 1987; WILLEMS et al., 1989). However, the high relatedness found between "[P.] setariae" and [P.] rubrilineans (r = 0,83) on the basis of their protein electrophoretic patterns, suggests a substantial degree of genome similarity between these two taxa. This should be investigated further by other techniques before a nomenclatural change is proposed.

The placement of [P.] pseudoalcaligenes subsp. citrulli in cluster I with [P.] rubrilineans and "[P.] setariae" is in agreement with the results of SCHAAD et al. (1978). These workers also found this watermelon bacterium to fit most closely into group III of the plant pathogenic pseudomonads as proposed by SANDS et al. (1970); a group that included [P.] rubrilineans and "[P.] setariae".

Except for [P.] avenae (NCPPB 2402) all the [P.] avenae strains clustered above r = 0,6. Because of the low correlation of strain NCPPB 2402 with the other [P.] avenae strains (r = 0,44) and because its authenticity is not known to have been verified (National Collection of Plant Pathogenic Bacteria, 1988), we question the identity of this strain and suggest that it should be re-examined.

In the [P.] avenae cluster (cluster II) no correlation was found between electrophoretic clustering and host plant or geographic origin.

As in the past, our results agreed well with those of other techniques, again accentuating the reliability and applicability of PAGE as a taxonomic tool in everyday phytobacterial practices.

The combination of our results with the results of other techniques, such as DNA-rRNA hybridization data, should contribute to a better understanding of the relationships within the acidovorans rRNA complex.

Key words: Phytopathogenic pseudomonads - Acidovorans rRNA
complex - Polyacrylamide gel electrophoresis -
Average linkage cluster analysis - Taxonomy

REFERENCES

BYNG, G.S., WHITAKER, R.J., GHERNA, R.L., JENSEN, R.A.: Variable enzymological patterning in tyrosine biosynthesis as a means of determining natural relatedness among the Pseudomonadaceae. J. Bacteriol. 144, 247-257 (1980)

DE VOS, P., DE LEY, J.: Intra- and intergeneric similarities of Pseudomonas and Xanthomonas ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 33, 487-509 (1983)

DE VOS, P., GOOR, M., GILLIS, M., DE LEY, J.: Ribosomal ribonucleic acid cistron similarities of phytopathogenic Pseudomonas species. Int. J. system. Bact. 35, 169-184 (1985)

DE VOS, P., VAN LANDSCHOOT, A., SEGERS, P., TYTGAT, R., GILLIS, M., BAUWENS, M., ROSSAU, R., GOOR, M., POT, B., KERSTERS, K., LIZZARAGA, P., DE LEY, J.: Genotypic relationships and taxonomic localization of unclassified Pseudomonas and Pseudomonas-like strains by deoxyribonucleic acid: ribosomal ribonucleic acid hybridizations. Int. J. system. Bact. 39, 35-49 (1989)

GAVINI, F., HOLMES, B., IZARD, D., BEJI, A., BERNIGUAD, A., JAKUBCZAK, E.: Numerical taxonomy of Pseudomonas alcaligenes, P. pseudoalcaligenes, P. mendocina, P. stutzeri, and related bacteria. Int. J. system. Bact. 39, 135-144 (1989)

GOOR, M., KERSTERS, K., GILLIS, M., THIELEMANS, S., DE LEY, J.: Taxonomy of the phytopathogenic Pseudomonas species belonging to the P. acidovorans rRNA branch, p. 37. In: Proceedings of the XIV Int. Congress of Microbiol., England. 1986.

INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY: Validation of the publication of new names and new combinations previously effectively published outside the IJSB. Lists no. 17-29. Int. J. system. Bact. 35-39 (1985-1989)

KERSTERS, K., DE LEY, J.: Identification and grouping of
bacteria by numerical analysis of their electrophorectic
protein patterns. J. gen. Microbiol. <u>87</u>, 333-342 (1975)

LAEMMLI, U.K.: Cleavage of structural proteins during the
assembly of the head of bacteriophage T4. Nature <u>227</u>, 680-685
(1970)

MOORE, W.E.C., CATO, E.P., MOORE, L.V.H.: Index of bacterial and
yeast nomenclatural changes published in the International
Journal of Systematic Bacteriology since the 1980 Approved
Lists of Bacterial Names (1 January 1980 - 1 January 1985).
Int. J. system. Bact. <u>35</u>, 382-407 (1985)

NATIONAL COLLECTION OF PLANT PATHOGENIC BACTERIA: <u>Pseudomonas</u>
Migula 1894, pp. 27-36. In: Catalogue of strains - National
Collection of Plant Pathogenic Bacteria, 2nd ed. England, Adas
laboratory services 1988

OYAIZU, H., KOMAGATA, K.: Grouping of <u>Pseudomonas</u> species on the
basis of cellular fatty acid compositions and the quinone
system with special reference to the existence of 3-hydroxy
fatty acids. J. gen. appl. Microbiol. <u>29</u>, 17-40 (1983)

PALLERONI, N.J.: Family I. <u>Pseudomonadaceae</u>, pp. 141-199. In:
Bergey's manual of systematic bacteriology (N.R. Krieg, J.G.
Holt, eds.), 9th ed., Vol. I. Baltimore, Williams and Wilkins
1984

SANDS, D.C., SCHROTH, M.N., HILDEBRAND, D.C.: Taxonomy of
phytopathogenic pseudomonads. J. Bact. <u>101</u>, 9-23 (1970)

SCHAAD, N.W., SOWELL, G., GOTH, R.W., COLWELL, R.R., WEBB, R.E.:
<u>Pseudomonas pseudoalcaligenes</u> subsp. <u>citrulli</u> subsp. nov. Int.
J. system. Bact. <u>28</u>, 117-125 (1978)

SKERMAN, V.B.D., MCGOWAN, V., SNEATH, P.H.A.: Approved lists of
   bacterial names. Int. J. system. Bact. 30, 225-420 (1980)

TAMAOKA, J., HA, D-M, KOMAGATA, K.: Reclassification of
   Pseudomonas acidovorans den Dooren de Jong 1926 and Pseudomonas
   testosteroni Marcus and Talalay 1956 as Comamonas acidovorans
   comb. nov. and Comamonas testosteroni comb. nov., with an
   emended description of the genus Comamonas. Int. J. system.
   Bact. 37, 52-59 (1987)

VAN ZYL, E., STEYN, P.L.: Differentiation of phytopathogenic
   Pseudomonas and Xanthomonas species and pathovars by numerical
   taxonomy and protein gel electrophoregrams. System. Appl.
   Microbiol. 13, 60-71 (1990)

VERA CRUZ, C.M., GOSSELé, F., KERSTERS, K., SEGERS, P., VAN DEN
   MOOTER, M., SWINGS, J., DE LEY, J.: Differentiation between
   Xanthomonas campestris pv. oryzae, Xanthomonas campestris pv.
   oryzicola and the bacterial "brown blotch" pathogen on rice by
   numerical analysis of phenotypic features and protein gel
   electrophoregrams. J. gen. Microbiol. 130, 2983-2999 (1984)

WILLEMS, A., GILLIS, M., KERSTERS, K., VAN DEN BROECKE, L., DE
   LEY, J.: Transfer of Xanthomonas ampelina Panagopoulos 1969 to
   a new genus, Xylophilus gen. nov., as Xylophilus ampelinus
   (Panagopoulos, 1969) comb. nov. Int. J. system. Bact. 37,
   422-430 (1987)

WILLEMS, A., BUSSE, J., GOOR, M., POT, B., FALSEN, E., JANTSEN,
   E., HOSTE, B., GILLIS, M., KERSTERS, K., AULING, G., DE LEY,
   J.: Hydrogenophaga, a new genus of hydrogen-oxidizing bacteria
   that includes Hydrogenophage flava comb. nov. (formerly
   Pseudomonas flava), Hydrogenophaga palleronii (formerly
   Pseudomonas palleroni), Hydrogenophaga pseudoflava (formerly
   Pseudomonas pseudoflava and "Pseudomonas carboxydoflava"), and
   Hydrogenophaga taeniospiralis (formerly Pseudomonas
   taeniospiralis). Int. J. system. Bact. 39, 319-333 (1989)

Table 1.   Strains used.

Species[a]	Strain[b]	Source, place and year of isolation
Comamonas acidovorans[c]	NCPPB 1967	Pharyngeal swab, 1966
[Pseudomonas] avenae	NCPPB 2398	Bromus catharticus, Japan, 1966
[Pseudomonas] avenae	NCPPB 2400	Euchlaena mexicana, Japan, 1967
[Pseudomonas] avenae	NCPPB 2402	Bromus inermis, Japan, 1967
[Pseudomonas] avenae	NCPPB 2403	Agropyron trichophorum, Japan, 1966
[Pseudomonas] avenae	NCPPB 3354	Oryza sativa seed, India, 1984
[Pseudomonas] avenae	NCPPB 3355	Oryza sativa seed, Brazil, 1984
[Pseudomonas] avenae	NCPPB 3356	Oryza sativa seed, Nepal, 1984
[Pseudomonas] avenae	NCPPB 3358	Oryza sativa seed, Turkey, 1984
[Pseudomonas] pseudo-alcaligenes subsp. citrulli	NCPPB 3244	Citrullus lanatus, USA, 1978
[Pseudomonas] rubrilineans	NCPPB 920	Saccharum officinarum, Reunion, 1960
"[Pseudomonas] setariae"[d]	NCPPB 1392	Oryza sativa, Japan, 1955

Legend to Table 1.   Strains used.

(a)   All misnamed taxa are indicated by brackets.

(b)   NCPPB: National Collection of Plant Pathogenic Bacteria,
       Harpenden, England.

(c)   [Pseudomonas] acidovorans was recently transferred to the
       genus Comamonas (TAMAOKA et al., 1987)

(d)   Name not retained on the Approved Lists of Bacterial Names
       (SKERMAN et al., 1980;   MOORE et al., 1985) or Validation
       Lists 17 to 29 (International Journal of Systematic
       Bacteriology, 1985-1989)

Fig. 1.   Dendrogram of the correlation coefficient, r, grouped
          by the unweighted average linkage cluster analysis,
          showing the taxonomic relationships between several
          phytopathogenic species of the acidovorans rRNA
          complex.

Fig. 1.
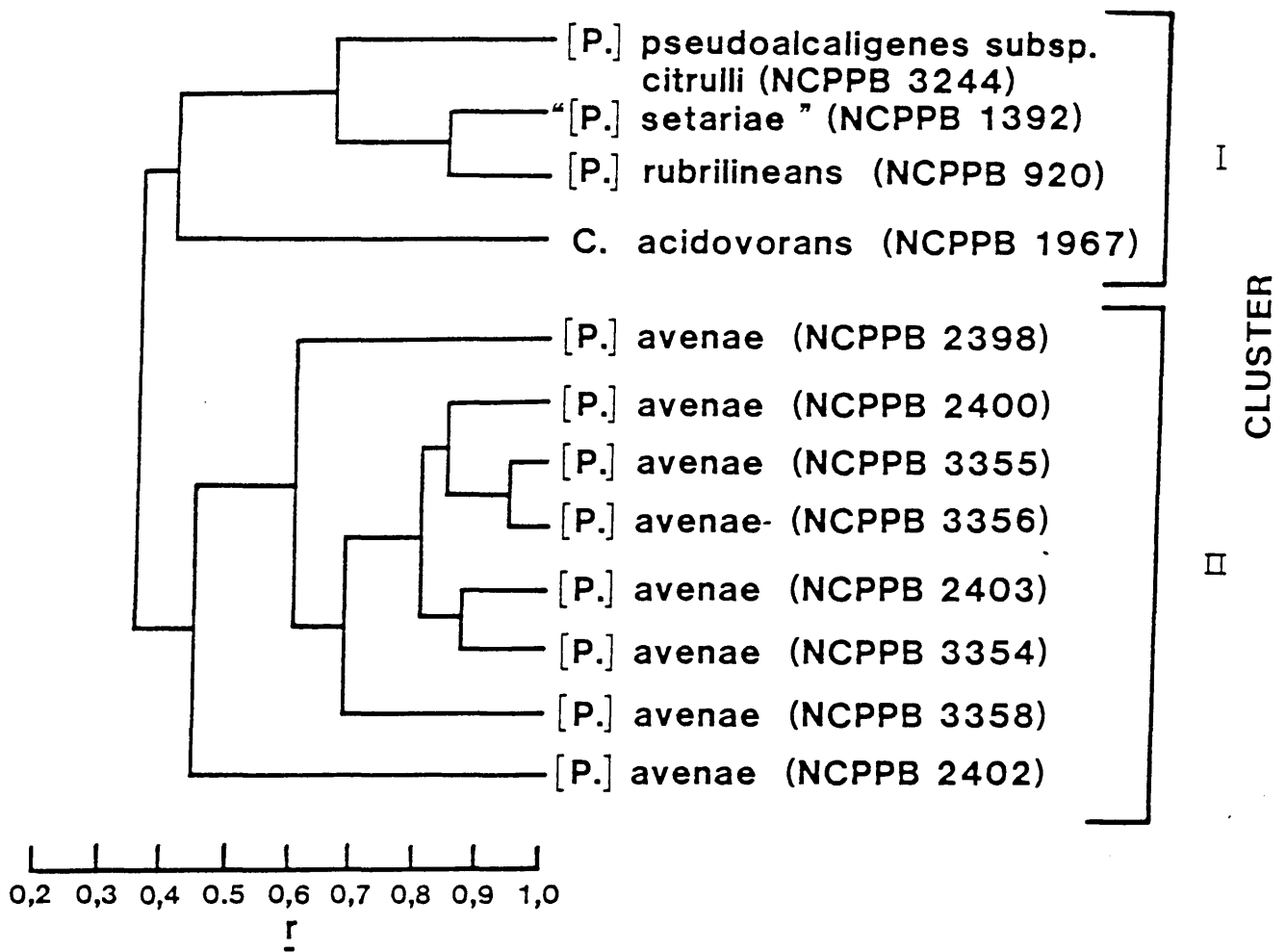
Request for an Opinion

Reinterpretation of the Taxonomic Position of <u>Xanthomonas maltophilia</u> and Taxonomic Criteria in this Genus

ELRITHA VAN ZYL and P.L. STEYN

Department of Microbiology and Plant Pathology,
University of Pretoria, Pretoria 0002,
Republic of South Africa

SUMMARY

The inclusion of "Pseudomonas" maltophilia in the genus Xanthomonas as Xanthomonas maltophilia is questioned in view of the significant differences between these two taxons. This reclassification is not acceptable if practical means of differentiation in this genus is considered. The proposed alteration of the genus description of Xanthomonas is also questionable because of the implications for everyday phytobacteriology. In view of the natural similarities, as well as the profound differences between X. maltophilia and Xanthomonas, it is proposed that a new genus is created for X. maltophilia, which could be placed together with Xanthomonas in a separate natural group.

INTRODUCTION

The taxonomic position of Xanthomonas maltophilia has been the subject of much debate (PALLERONI et al., 1973; IKEMOTO et al., 1980, PALLERONI, 1981; STARR, 1981; SWINGS et al., 1983; DE VOS and DE LEY, 1983; BRADBURY, 1984; PALLERONI, 1984; JOHNSON and PALLERONI, 1989). Initially isolated from pleural fluid in 1943 and named "Bacterium bookerii", this ubiquitous bacterium was reclassified as Pseudomonas maltophilia by HUGH and RYSCHENKOW (1961). As "P". maltophilia and species of Xanthomonas share a substantial level of rRNA homology (PALLERONI et al., 1973), it appeared appropriate to assign this species to the genus Xanthomonas (PALLERONI, 1984). As a number of arguments in the literature also support this view, SWINGS et al. (1983) proposed the transfer of P. maltophilia Hugh 1981 to the genus Xanthomonas as Xanthomonas maltophilia (Hugh 1981) comb. nov.

In view of the significant differences between these two taxons, this reclassification remains questionable (PALLERONI, 1986), partly because of the redefinition of the genus Xanthomonas that will be required to accommodate X. maltophilia (BRADBURY, 1984; PALLERONI, 1984).

As our own results (VAN ZYL and STEYN, 1990) also argue against inclusion of X. maltophilia in the genus Xanthomonas, the purpose of this paper is to question some of the assumptions made by SWINGS et al. (1983), to summarise the latest information on this subject, and to request an opinion.

The following arguments proceed from the reclassification proposed by SWINGS et al. (1983) and should be considered together with their corresponding discussions:

DNA-rRNA HYBRIDIZATIONS: The taxonomic value and reliability of this technique for the classification of bacteria on generic and suprageneric levels have been demonstrated by several workers in this field (DE SMEDT and DE LEY, 1977; DE LEY et al., 1978; DE SMEDT et al., 1980; GILLIS and DE LEY, 1980; DE VOS and DE LEY, 1983; DE VOS et al., 1985). However, from the reclas= sification proposed by SWINGS et al. (1983), the following are not clear: although SWINGS et al. (1983) refer to an extensive DNA-rRNA hybridization study of the genus Pseudomonas by De Vos and De Ley, different $T_{m(\bullet)}$ ranges are reported by these authors for exactly the same Xanthomonas strains. SWINGS et al. (1983) reported a range of 78,8 to 81,5°C, while DE VOS and DE LEY (1983) found it to be 80,0 to 81,5°C for the same 27 Xanthomonas strains under identical conditions and using the same reference rRNA. In the case of SWINGS et al. (1983) the range determined for X. maltophilia (76,5 to 78,0°C) (DE VOS and DE LEY, 1983; SWINGS et al., 1983) will therefore almost overlap with that of Xanthomonas, whereas DE VOS and DE LEY (1983) found X. maltophilia to be removed from Xanthomonas at a $T_{m(\bullet)}$ of 3°C under these conditions. This places the X. maltophilia cluster close to the genus Xanthomonas, but still out of range. Also

when using reference rRNA from three <u>Pseudomonas</u> type strains, <u>X</u>. <u>maltophilia</u> was located close to <u>Xanthomonas</u>, but never included in the same similarity cluster (DE VOS and DE LEY, 1983).

As the amount of hybrid formation depends not only on the actual sequence homology, but also on the size of the genome, its state of replication and the number of rRNA cistrons per genome, rRNA similarity should be expressed by two parameters, percent rRNA binding and melting point [$T_{m(e)}$] of DNA-rRNA hybrids (KANDLER and SCHLEIFER, 1980). SWINGS et al. (1983) did not mention the % rRNA binding in this case. Although the percentage of rRNA binding might not be a direct measure of rRNA cistron homology, it is a useful parameter to separate taxa with the same $T_{m(e)}$ value (DE VOS et al., 1985), in this case removing <u>X</u>. <u>maltophilia</u> even further from the extremely tight <u>Xanthomonas</u> cluster when plotted on a similarity map (DE VOS and DE LEY, 1983).

As DNA-rRNA hybridizations were the first indicators of a possible transfer of "<u>P</u>". <u>maltophilia</u> to the genus <u>Xanthomonas</u> (PALLERONI et al., 1973), it is of interest to note the opinion of WOESE et al. (1984) on the DNA-rRNA approach. According to them the DNA-rRNA approach offers no advantage over DNA-DNA hybridization (which already revealed low levels of DNA similarity among <u>Pseudomonas</u> species (JOHNSON and PALLERONI, 1989)) when it comes to groupings at the generic level, and has the disadvantage that it samples only one or two genes. In their opinion the DNA-rRNA approach should soon become obsolete as it offers neither the precision nor the extent of data obtainable with sequencing approaches for example.

DNA-DNA HOMOLOGY: At present DNA-DNA homology is widely accepted as an expression of genetic relatedness (IKEMOTO et al., 1980). Membrane competition experiments, however, revealed very low levels of DNA similarity among <u>Pseudomonas</u> species of the five different rRNA subgroups (JOHNSON and PALLERONI, 1989).

Interspecies similarity values are in the 5 to 20% range, with the average DNA similarity values between species belonging to different rRNA similarity clusters even lower (not more than 1 to 2%) (JOHNSON and PALLERONI, 1989). This presents a practical problem for the classification of species belonging to this group of bacteria, because, although it is not always clear at what point on the homology scale the lines for interspecies and intragenus relatedness should be drawn (GARDNER and KADO, 1972), it is generally accepted that the genosubspecies would be those strains that have a minimum of 60 to 70% DNA homology (JOHNSON, 1973; VAUTERIN et al., 1990). Unfortunately, there is currently no satisfactory phylogenetic definition of a genus (WAYNE et al., 1987). For X. maltophilia the percentage similarity with labeled DNA from X. campestris strains range from 22 to 37% (IKEMOTO et al., 1980) or 1 to 16% with the S1 nuclease methodology (JOHNSON and PALLERONI, 1989). Such low values are at the border of or below the sensitivity of this method (SWINGS et al., 1983) and very difficult to interpret.

The low levels of DNA similarity between Pseudomonas species has far reaching implications for taxonomy and phylogenetic relationships in this group, when it is considered that the DNA-DNA approach actually represents (an average of) the entire genome, and not merely a few genes therein (WOESE et al., 1984). For classification purposes it is now also considered necessary to calibrate techniques for the differentiation of strains against DNA-DNA hybridization, the conventional method used for the delineation of species (VAUTERIN et al., 1990).

DNA-DNA hybridization data divided rRNA group V strains into two subgroups: a X. maltophilia subgroup and a X. campestris subgroup, with substantial heterogeneity among the strains in each subgroup (JOHNSON and PALLERONI, 1989). IKEMOTO et al. (1980) also observed heterogeneity among X. maltophilia strains and delineated five clusters on the basis of DNA-DNA homology. There was also a difference between methionine-requiring and nonrequiring strains of X. maltophilia with respect to DNA-DNA homology (IKEMOTO et al., 1980).

GUANINE-PLUS-CYTOSINE CONTENTS: The G + C range determined for X. maltophilia (63-67,5 mol%) fit well into the Xanthomonas range (63-71 mol%) (BRADBURY, 1984), and although it will also fit into the Pseudomonas range (58-70 mol%) (PALLERONI, 1984), these values are not inconsistent with inclusion of X. maltophilia in Xanthomonas. It is, however, also important to bear in mind that although these mol% G + C values are closely similar for related organisms, the reverse is not true: two organisms with similar mol% G + C are not necessarily related (DE LEY, 1968).

COMPARATIVE ENZYMOLOGY: According to SWINGS et al. (1983), "both P. maltophilia and Xanthomonas are unusual in that they lack NADP-linked dehydrogenases and possess NAD-specific prephenate and arogenate dehydrogenases for tyrosine biosynthesis". Referring to the original article on this topic (BYNG et al., 1980) it is learnt that rRNA homology groups I, IV and V all lack activity for arogenate/NADP dehydrogenase, while groups I and V also lack prephenate/NADP dehydrogenase. As both groups I and V furthermore possess NAD-specific prephenate and arogenate dehydrogenasses for tyrosine biosynthesis (BYNG et al., 1980), X. maltophilia and Xanthomonas are not so unique in this regard. Groups I and V are separated by comparison of the relative allosteric sensitivities of NAD-dependent dehydrogenases (BYNG et al., 1980). Unfortunately results for X. maltophilia were not included in the comparison of the sensitivities of the NAD-dependent dehydrogenases to tyrosine inhibition (Table 5 - BYNG et al., 1980) and was only one Pseudomonas species (P. gardneri) grouped with all the Xanthomonas species studied (BYNG et al., 1980).

The control pattern for the enzyme 3-deoxy-D-arabinoheptulo-sonate-7-phosphate (DAHP) synthetase is a useful indicator of taxonomic relationship at about the level of genus (WHITAKER et al., 1981). The occurrence of this unique control mechanism in X. maltophilia as well as species of Xanthomonas indeed supports a close taxonomic relationship between these two taxa (WHITAKER

et al., 1981; SWINGS et al., 1983), but it has to be remembered that the comparative allostery of DAHP synthetase alone does not unambiguously establish group placement (WHITAKER et al., 1981).

UBIQUINONES: SWINGS et al. (1983) stated that both X. maltophilia and Xanthomonas contain ubiquinones with eight isoprene units (Q-8), whereas all other Pseudomonas strains tested generally contain ubiquinones with nine isoprene units (Q-9) as major components. In our opinion the above statement does not reflect the true situation correctly. OYAIZU and KOMAGATA (1983) examined the quinone system of 75 Pseudomonas strains and divided them into nine groups according to this characteristic, as well as fatty acid composition. Of the nine groups determined only group I (corresponding to rRNA homology group I) species contain a Q-9 system, while five groups of named pseudomonads contain Q-8 quinone systems. This include a P. solanacearum group (rRNA group II), a "P". acidovorans group (rRNA group III), X. maltophilia (rRNA group V), P. avenae and P. palleronii (rRNA group III). The three other groups contain Q-10 systems (OYAIZU and KOMAGATA, 1983).

Based on ubiquinones, the X. maltophilia-Xanthomonas group is not as unique as implied by SWINGS et al. (1983).

CELLULAR FATTY ACID COMPOSITION: Quantitative differences were found between the fatty acids of X. maltophilia strains that did not require methionine and methionine-requiring strains of this species, although they share almost the same kinds of fatty acids (IKEMOTO et al., 1980). Based on cellular fatty acid compositions a similarity value of 82% was calculated for the two groups (IKEMOTO et al., 1980). From the data by IKEMOTO et al. (1980) it is interesting to note that X. maltophilia strains which do not require methionine also share relatively high mean percentages of i-$C_{16:0}$ and $C_{17:0}$ fatty acids with the two Xanthomonas strains (X. oryzae excluded), values considerably higher than the percentages determined for methionine-requiring strains of X. maltophilia. X. oryzae has a fatty acid

composition quite different from that of X. maltophilia (IKEMOTO et al., 1980).

PHAGE TYPING: Phage typing studies provided evidence for a relationship between X. maltophilia and Xanthomonas (SWINGS et al., 1983). This technique was, however, not very successful as a taxonomic tool in Xanthomonas, mainly because of lack of specificity (GARRETT, 1982; BRADBURY, 1984).

In addition temperate phages have been found in X. maltophilia (PALLERONI, 1984), while nothing to this nature has been reported for Xanthomonas.

GROWTH AND PIGMENTS: The colonies of Xanthomonas species are distinctively yellow in colour, and this pigmentation is very important in their presumptive identification (STARR, 1981). It is suggested that these xanthomonadins might well serve as adequate chemotaxonomic markers for the genus Xanthomonas (STARR, 1981). X. maltophilia cultures do not show the yellow mucoid growth typical of Xanthomonas, and its pigments are not xanthomonadins or closely related to the xanthomonadins already explored (STARR, 1981; SWINGS et al., 1983; PALLERONI, 1984).

Nonpigmented Xanthomonas strains do exist, but evidence in the literature indicate that pigmented forms exist for all the nonpigmented Xanthomonas species which occur naturally (SABET, 1959; ROBBS et al., 1972; STARR and STEPHENS, 1964; ROBBS et al., 1974).

SWINGS et al. (1983) report that some X. maltophilia strains characteristically form brownish grey water-soluble pigments, also observed in six pathovars of X. compestris. GILARDI (1971) is of the opinion that the brown coloration of the culture medium associated with X. maltophilia and previously described as a pigment, is probably due to a secondary chemical reaction among extracellular products which react to form the brown colour.

When grown on media containing usable carbohydrates, most
Xanthomonas strains produce characteristic extracellular
polysaccarides or xanthan gums, resulting in very mucoid colonies
(SWINGS et al., 1983; BRADBURY, 1984). The growth of X.
maltophilia is shiny, but not mucoid (SWINGS et al., 1983). This
presents a practical problem, as slime formation has some value
in screening Xanthomonas cultures (DEY, 1962).

NICHES: All species currently included in the genus
Xanthomonas are highly specialised plant pathogens (BRADBURY,
1984), while at present nothing is known about the
phytopathogenic capacity of X. maltophilia strains (SWINGS et
al., 1983). Most X. maltophilia strains are isolated from
clinical specimens - X. maltophilia is the second most frequently
isolated Pseudomonas species in the clinical laboratory
(PALLERONI, 1984). Strains of this species appear to be
opportunistic human pathogens (PALLERONI, 1984). There is
presently no evidence of any Xanthomonas species associated with
human infections.

X. maltophilia strains are also found in frozen food, milk and
water, while X. maltophilia predominates over other pseudomonads
in the rhizospheres of several cultivated plants, probably due to
the excretion of S-containing amino acids by the roots
(PALLERONI, 1984). For Xanthomonas species, however, the minimum
growth temperature is above 5°C (BRADBURY, 1984). Xanthomonads
have occasionally been detected in runn-off water and ditches
around fields of infected plants, but results indicate that
survival in this situation would be short (BRADBURY, 1984). For
Xanthomonas species, survival in soil saprophytically is unusual
(BRADBURY, 1984). Two pathovars of X. campestris are, however,
known to spend the interseasonal time epiphytically (BRADBURY,
1984).

OTHER MORPHOLOGICAL, PHYSIOLOGICAL, AND BIOCHEMICAL
CHARACTERISTICS: From the literature it is known that X.
maltophilia and Xanthomonas share a number of morphological,

.

physiological and biochemical characteristics (SWINGS et al., 1983). SWINGS et al. (1983) calculated a simple matching similarity coefficient (S_{SM}) of approximately 75% between these two taxons from the available phenotypic data.

In spite of a high similarity value (S_{SM}) of 79% calculated for X. maltophilia in a phenotypic study using 53 standard phenotypic tests, X. maltophilia was less related to the Xanthomonas group than the majority of Pseudomonas strains studied (VAN ZYL and STEYN, 1990). This was confirmed by the results of the numerical analysis of electrophoretic protein patterns of the same strains, where a correlation of r = 0,65 was determined between X. maltophilia and the bigger Pseudomonas/ Xanthomonas cluster (VAN ZYL and STEYN, 1990).

The number of polar flagella is an important taxonomic character (PALLERONI, 1984). The existence of a single polar flagellum is an important characteristic of Xanthomonas. Very rarely cells occur with two polar flagella (BRADBURY, 1984). On the other hand X. maltophilia is multitrichously flagellated (SWINGS et al., 1983; PALLERONI, 1984).

At present no pili or fimbriae have been reported for Xanthomonas (BRADBURY, 1984), while polarly inserted pili or fimbriae were reported for X. maltophilia (PALLERONI, 1984).

According to the present generic definition of Xanthomonas, no nitrate reducing occurs (BRADBURY, 1984). X. maltophilia strains reduce nitrates to nitrites (KOMAGATA et al., 1974; SWINGS et al., 1983).

STARR (1946) and DYE (1962) reported that asparagine is inadequate as a sole source of carbon and nitrogen for xanthomonads. BRADBURY (1984) included this property in the definition of the genus Xanthomonas and this is presently used as a diagnostic test for Xanthomonas, since yellow Enterobacteriaceae and many Pseudomonas species will grow with

asparagine as sole source of both carbon and nitrogen (BRADBURY, 1984). X. maltophilia (NCPPB 1974) also grows vigorously under these conditions (VAN ZYL and STEYN, 1990).

Some of the characteristics shared by X. maltophilia and Xanthomonas according to SWINGS et al. (1983) are acid production from (amongst others) lactose, fructose and maltose, as well as hydrolysis of aesculin. According to BRADBURY (1984) there is actually a variation in the reactions: for acid production from lactose and maltose three Xanthomonas species gave negative reactions, while 11-89% of the X. campestris strains were positive; for acid production from fructose, two Xanthomonas species gave negative reactions, while three species were positive. Hydrolysis of aesculin also gave a varied reaction, with three species positive and one Xanthomonas species negative (BRADBURY, 1984).

Other differences between X. maltophilia and Xanthomonas include the production of lysine decarboxylase, starch hydrolysis and resistance to antibiotics, dyes and metals (SWINGS et al., 1983).

From the above it is clear that profound differences exist between these two taxons; differences which are not without substantial practical implications for the already complex taxonomic structure and identification system in Xanthomonas.

ADDITIONAL REMARKS: Since the publication of the proposal for the transfer of "P". maltophilia to the genus Xanthomonas (SWINGS et al., 1983), the following appeared in favour of or as an argument against this inclusion:

Oligonucleotide cataloging of 16S rRNA: S$_{AB}$ analysis of oligonucleotide cataloging showed X. maltophilia and "its close relatives (i.e. the xanthomonads)" to cluster peripherally with the fluorescent pseudomonas group (P. fluorescens, P. aeruginosa, P. syringae etc.) in particular (WOESE et al., 1984). Catalogs

of the X. maltophilia group, however, also have a remarkable number of oligonucleotides in common with group II species ("P. acidovorans, P. testosteroni, P. cepacia etc.) (WOESE et al., 1984).

Respiratory chain: Difference spectra at liquid air temperature showed a slight shoulder for Xanthomonas at 549 nm, suggesting a very small content of cytochrome c. The difference spectra for P. syringae and X. maltophilia are very similar, but do not show any noticeable peak or shoulder at or near 549 nm (BRADBURY, 1984).

Growth factor requirement: Many Xanthomonas strains require growth factors, usually amino acids such as methionine and/or glutamic acid. Nicotinic acid is occasionally required (BRADBURY, 1984). X. maltophilia has a requirement for methionine, although strains of X. maltophilia which do not require methionine have been described (IKEMOTO et al., 1980). These methionine-requiring and -nonrequiring strains of X. maltophilia differ from each other with respect to phenotypic characteristics, cellular fatty acid composition, DNA base composition and DNA-DNA hybridization, and are separable into two clusters on the basis of methionine requirement and some other properties (IKEMOTO et al., 1980). It is also noteworthy that the requirement for methionine depended upon the carbon compounds employed (IKEMOTO et al., 1980). Although it is likely that the genus Pseudomonas will be reserved in the future for species not requiring growth factors, there are also a few Pseudomonas species that require the addition of organic growth factors. P. diminuta for example also needs methionine, while growth factor-requiring strains of P. caryophylli and P. syringae are occasionally encountered in nature (PALLERONI, 1984).

Metabolism of aromatic compounds: On the basis of the dehydratases of the two pathways to phenylalanine, five rRNA/DNA hybridization groups were distinguished. X. maltophilia was assigned to group V together with other Xanthomonas strains.

Group V could, however, not be distinguished from group Ib us·ing
these enzymes (BRADBURY, 1984).

Glycoside hydrolases: HAYWARD (1977) demonstrated the activity
of α-glucosidase, β-glucosidase, β-galactosidase and β-xylosidase
in most of the 39 Xanthomonas strains examined, as well as in X.
maltophilia ("P". maltophilia) alone among the Pseudomonas
species.

Cell walls: X. maltophilia and Xanthomonas share a high
content of rhamnose, low content of 2-keto-3-deoxyoctonate,
presence of D-galacturonic acid, and the absence of heptoses in
their cell walls. All the Xanthomonas strains examined so far
for the above characteristics, however, belonged only to X.
campestris (BRADBURY, 1984). An interesting feature of the
lipopolysaccharide of X. maltophilia is the presence of a pentose
derivative that has been identified as 3-0-methyl-L-xylose, thus
far not found in other bacteria with the exception of
Rhodopseudomonas viridis (PALLERONI, 1984).

Exoproducts: A high degree of similarity (exceeding 90%)
between the electrophoregrams of the exoproducts of 31
Pseudomonas strains, including (amongst others) X. maltophilia,
P. aeruginosa, P. fluorescens, P. alcaligenes etc., has been
shown by polyacrylamide gel electrophoresis (VOLCHKEVICH and
DEGTEVA, 1989).

In conclusion, we find the following features not inconsistent
with inclusion of X. maltophilia in Xanthomonas: guanine-plus-
cytosine contents, comparative enzymology, ubiquinones, cellular
fatty acid composition, phage typing, oligonucleotide cataloging
of 16S rRNA and glycoside hydrolase activity, although some
features are not always as unique as implied. The following are,
however, presently sufficient reasons to exclude X. maltophilia
from Xanthomonas: the lack of xanthomonadins, xanthan gums and
plant pathogenicity, its association with human infections,
saprophytic survival in several niches, and its occurring free
living in nature; also on the basis of the number of polar

flagella, the existence of pili or fimbriae, nitrate reduction, asparagine utilisation, low correlation in electrophoretic protein patterns, the number of properties shared also with Pseudomonas species (respiratory chain, exoproducts, the dehydratases of the two pathways to phenylalanine and growth factor requirement in some cases), the existence of methionine-nonrequiring strains of X. maltophilia, as well as several other differences mentioned, it does not seem appropriate or satisfactory to include X. maltophilia in Xanthomonas. DNA-DNA and DNA-rRNA hybridization results can be interpreted in various ways, but to our mind it also points to inclusion of the two taxons in a natural group as "close relatives", rather than inclusion in the same genus.

SWINGS et al. (1983) proposed an improved definition of the genus Xanthomonas, namely that "all Xanthomonas strains tested fall in the $T_{m(e)}$ range from 76 to 81°C when their DNAs are hybridized with labelled rRNA from type strain X. campestris pv. campestris NCPPB 528". It is, however, not clear to what extent this $T_{m(e)}$ range has been lowered mainly to include X. maltophilia.

In our view the question of the reclassification of X. maltophilia is closely linked to the whole taxonomic situation in the genus Xanthomonas. Presently there are many arguments in favour of a phylogeneticaly based system of classification rather than a determinative one. It will, however, be a major setback for everyday phytobacteriology, where speed, cost effectiveness and the availability of sophisticated apparatus are often issues, if an improved definition of the genus Xanthomonas is based upon characters of phylogenetic value which can not be readily determined or repeated by less equipped laboratories. VAUTERIN et al. (1990) are for example of the opinion that in a general taxonomic environment there is no reason to assign crucial importance to a single phenotypic feature such as plant pathogenicity. Whether it is justified or not, plant pathogenicity and host specificity play a very important role in

the presumptive identification of xanthomonads by plant pathologists, and provision must be made for this feature, as well as several other practical means of differentiation, in the proposed improved definition of <u>Xanthomonas</u>. Although modern bacterial systematists can not be expected to work with an artificial type of classification for practical purposes only, a natural one without practical implication is also not acceptable (VAUTERIN et al., 1990). It is completely impracticable to define genera solely on the basis of phylogenetic data. Genera need to be characterized by using phenotypic properties, even if the choice of phenotypic markers might change given the development of better tests (MURRAY et al., 1990). The same applies to <u>X</u>. <u>maltophilia</u>. Although evidence of a more phylogenetic nature indicate a close relationship between <u>X</u>. <u>maltophilia</u> and <u>Xanthomonas</u>, its inclusion in <u>Xanthomonas</u> is not acceptable on practical grounds. In order to make provision for the natural similarities, as well as the significant differences discussed here, it is proposed that a separate genus is created for <u>X</u>. <u>maltophilia</u>, which could be placed with <u>Xanthomonas</u> in the same natural group.

Key words: <u>Xanthomonas</u> - <u>X</u>. <u>maltophilia</u> - Genotypic and phenotypic features - Taxonomy

REFERENCES

BRADBURY, J.F.: Genus II. Xanthomonas, pp. 199-120. In: Bergey's manual of systematic bacteriology (N.R. KRIEG, J.G. HOLT, eds.), 9th ed., vol. 1. Baltimore, Williams and Wilkens 1984

BYNG, G.S., WHITAKER, R.J., GHERNA, R.L., JENSEN, R.A.: Variable enzymological patterning in tyrosine biosynthesis as a new means of determining natural relatedness among Pseudomonadaceae. J. Bact. 144, 247-257 (1980)

DE LEY, J.: DNA base composition and hybridization in the taxonomy of phytopathogenic bacteria. Ann. Rev. Phytopath. 6, 63-90 (1968)

DE LEY, J., SEGERS, P., GILLIS, M.: Intra- and intergeneric similarities of Chromobacterium and Janthinobacterium ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 28, 154-168 (1978)

DE SMEDT, J., BAUWENS, M., TYTGAT, R., DE LEY, J.: Intra- and intergeneric similarities of ribosomal ribonucleic acid cistrons of free-living, nitrogen-fixing bacteria. Int. J. system. Bact. 30, 106-122 (1980)

DE SMEDT, J., DE LEY, J.: Intra- and intergeneric similarities of Agrobacterium ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 27, 222-240 (1977)

DE VOS, P., DE LEY, J.: Intra- and intergeneric similarities of Pseudomonas and Xanthomonas ribosomal ribonucleic acid cistrons. Int. J. system. Bact. 33, 487-509 (1983)

DE VOS, P., GOOR, M., GILLIS, M., DE LEY, J.: Ribosomal ribocleic acid cistron similarities of phytopapthogenic Pseudomonas species. Int. J. system. Bact. 35, 169-184 (1985)

DYE, D.W.: The inadequacy of the usual determinative tests for the identification of Xanthomonas spp. N.S. J. Sci. 5, 393-416 (1962)

GARDNER, J.M., KADO, C.I.: Comparative base sequence homologies of the deoxyribonucleic acids of Erwinia species and other Enterobacteriaceae. Int. J. system. Bact. 22, 201-209 (1972)

GARRETT, C.M.E.: Bacterial diseases of food plants - an overview, pp. 115-132. In: Bacteria and plants (M.E. RHODES-ROBERTS, F.A. SKINNER, eds.). London, Academic Press 1982

GILARDI, G.L.: Characterization of Pseudomonas species isolated from clinical specimens. Appl. Microbiol. 21, 414-419 (1971)

GILLIS, M., DE LEY, J.: Intra- and intergeneric similarities of the ribosomal ribonucleic acid cistrons of Acetobacter and Gluconobacter. Int. J. system. Bact. 30, 7-27 (1980)

HAYWARD, A.C.: Occurrence of glycoside hydrolases in plant pathogenic and related bacteria. J. appl. Bact. 42, 407-411 (1977)

HUGH, R., RYSCHENKOW, E.: Pseudomonas maltophilia, an Alcaligenes-like species. J. gen. Microbiol. 26, 123-132 (1961)

IKEMOTO, S., SUZUKI, K., KANEKO, T., KOMAGATA, K.: Characterization of strains of Pseudomonas maltophilia which do not require methionine. Int. J. system. Bact. 30, 437-447 (1980)

JOHNSON, J.L.: Use of nucleic acid homologies in the taxonomy of anaerobic bacteria. Int. J. system. Bact. 23, 308-315 (1973)

JOHNSON, J.L., PALLERONI, N.J.: Deoxyribonucleic acid similarities among Pseudomonas species. Int. J. system. Bact. 39, 230-235 (1989)

KANDLER, O., SCHLEIFER, K-H.: Systematics of bacteria, pp. 234-237. In: Progress in botany (H. ELLENBERG, K. ESSER, K. KUBITZKI, E. SCHNEPF, H. ZIEGLER, eds.), vol. 42. Berlin, Springer-Verlag 1980

KOMAGATA, K., YABUUCHI, E., TAMAGAWA, Y., OHYAMA, A.: Pseudomonas melanogena Ilizuka and Komagata 1963, a later subjective synonym of Pseudomonas maltophilia Hugh and Ryschenkow 1960. Int. J. system. Bact. 24, 242-247 (1974)

MURRAY, R.G.E., BRENNER, D.J., COLWELL, R.R., DE VOS, P., GOODFELLOW, M., GRIMONT, P.A.D., PFENNIG, N., STACKEBRANDT, E., ZAVARZIN, G.A.: Report of the ad hoc committee on approaches to taxonomy within the proteobacteria. Int. J. system. Bact. 40, 213-215 (1990)

OYAIZU, H., KOMAGATA, K.: Grouping of Pseudomonas species on the basis of cellular fatty acid compositions and the quinone system with special reference to the existence of 3-hydroxy fatty acids. J. gen appl. Microbiol. 29, 17-40 (1983)

PALERONI, N.J.: Introduction to the family Pseudomonadaceae, pp. 655-665. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (M.P. STARR, H., STOLP, H.G. TRÜPER, A. BARLOWS, H.G. SCHLEGEL, eds.). Berlin, Springer-Verlag 1981

PALLERONI, N.J.: Family I. Pseudomonadaceae, pp. 141-199. In: Bergey's manual of systematic bacteriology (N.R. KRIEG, J.G. HOLT, eds.), 9th ed., vol. I. Baltimore, Williams and Wilkins 1984.

PALLERONI, N.J.: Taxonomy of the pseudomonads. pp. 3-25. In: The bacteria: the biology of Pseudomonas (J.R. SOKATCH, ed.). London, Academic Press 1986

PALLERONI, N.J., KUNISAWA, R., CONTOPOULOU, R., DOUDOROFF, M.: Nucleic acid homologies in the genus Pseudomonas. Int. J. system. Bact. 23, 333-339 (1973)

ROBBS, C.F., RIBEIRO, R. de L.D., KIMURA, O., AKIBA, F.: Variaqoes em Xanthomonas manihotis (Arthaud-Berthet) Sstarr. Rev. Soc. Bras. Fitopatol. 5, 67-75 (1972)

ROBBS, C.F., RIBEIRO, R. de L.D., KIMURA, O.: Sobre a posiqao taxonomica de Pseudomonas mangiferaeindicae Patel et al. 1948, agente causal da "mancha bacteriana" das folhas da mangueira (Mangifera infica L.). Arq. Univ. Fed. Rural Rio de Janeiro 4, 11-14 (1974)

SABET, K.A.: Studies in the bacterial diseases of Sudan crops. II. Bacterial leaf blight disease of castor (Ricinus communis L.). Ann. appl. Biol. 47, 49-56 (1959)

STARR. M.P.: The nutrition of phytopathogenic bacteria. I. Minimal requirements of the genus Xanthomonas. J. Bact. 51, 131-143 (1946)

STARR, M.P.: The genus Xanthomonas, pp. 742-763. In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria (M.P. STARR, H. STOLP, H.G. TRÜPER, A., BARLOWS, H.G. SCHLEGEL, eds.). Berlin, Springer-Verlag 1981

STARR, M.P., STEPHENS, W.L.: Pigmentation and taxonomy of the genus Xanthomonas. J. Bact. 87, 293-302 (1964)

SWINGS, J., DE VOS, P., VAN DEN MOOTER, M., DE LEY, J.: Transfer of Pseudomonas maltophilia Hugh 1981 to the genus Xanthomonas as Xanthomonas maltophilia (Hugh 1981) comb. nov. Int. J. system. Bact. 33, 409-413 (1983)

VAN ZYL, E., STEYN, P.L.: Differentiation of phytopathogenic Pseudomonas and Xanthomonas species and pathovars by numerical taxonomy and protein gel electrophoregrams. System. Appl. Microbiol. 13, 60-71 (1990)

VAUTERIN, L., SWINGS, J., KERSTERS, K., GILLIS, M., MEW, T.W., SCHROTH, M.N., PALLERONI, N.J., HILDEBRAND, D.C., STEAD, D.E., CIVEROLO, E.L., HAYWARD, A.C., MARAîTE, H., STALL, R.E., VIDAVER, A.K., BRADBURY, J.F.: Towards an improved taxonomy of Xanthomonas. Int. J. system. Bact. 40, 312-316 (1990)

VOLCHKEVICH, Z.A., DEGTEVA, G.K.: Electrophoretic analysis of Pseudomonas exoproducts. Zh. Mikrobiol. Epidemiol. Immunobiol. 8, 24-28 (1989)

WAYNE, L.G., BRENNER, D.J., COLWELL, R.R., GRIMONT, P.A.D., KANDLER, O., KRICHEVSKY, M.I., MOORE, L.H., MOORE, W.E.C., MURRAY, R.G.E., STACKEBRANDT, E., STARR, M.P., TRÜPER, H.G.: Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. system. Bact. 37, 463-464 (1987)

WHITAKER, R.J., BYNG, G.S., GHERNA, R.L., JENSEN, R.A.: Comparative allostery of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase as an indicator of taxonomic relatedness in pseudomonad genera. J. Bact. 145, 752-759 (1981)

WOESE, C.R., BLANZ, P., HAHN, C.M.: What isn't a pseudomonad: the importance of nomenclature in bacterial classification. System. Appl. Microbiol. 5, 179-195 (1984)