Phenotypic and genotypic diversity of fluorescent pseudomonads isolated from field-grown sugar beet

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A sample of 30 fluorescent pseudomonads isolated from the phyllosphere of sugar beet throughout a single growing season and shown to be closely related on the basis of fatty acid methyl ester (FAME) analysis was subjected to detailed phenotypic and genotypic characterization. Phenotypic traits were assessed on the basis of biochemical properties, assimilation of sole carbon sources, FAME analysis, organic pyrolysate content (MS-pyrolysis), and total cellular protein profiles. With the exception of total cellular protein profiles, numerical analysis of the data revealed two main clusters, each of which was divided into several subclusters. Numerical analysis of total cellular protein data failed to differentiate isolates into two main clusters, but nevertheless grouped isolates into six subclusters. On the basis of biochemical and carbon source assimilation profiles, 19 isolates were identified as *Pseudomonas fluorescens* biovar V, eight isolates as *P. fluorescens* biovar III and three isolates as *P. syringae* pathovar *syringae*. In general, all methods of phenotypic analysis grouped isolates according to time of sampling and leaf type. Genome analysis was undertaken by pulsed-field gel electrophoresis (PFGE) of *PacI*, *SpeI*, *SwaI* and *XbaI* macrorestriction fragments and revealed the presence of eight distinct genomic (clonal) groups. These groups correlated closely with the clusters generated by numerical analysis of phenotypic data, but there was no correlation between macrorestriction fragment profile and isolate identification; in fact the variation in macrorestriction fragment patterns within *P. fluorescens* biovars was as great as the variation detected between biovars, and between *P. fluorescens* and *P. syringae*. Statistical evaluation of macrorestriction fragment patterns revealed two examples of recent strain divergence: one was due to the presence of a 400 kbp plasmid within one isolate of a collection of nine otherwise genomically identical isolates, and the other was observed between two phenotypically similar isolates sampled 220 d apart. Genetic variation was expressed in terms of nucleotide diversity (π) and pairwise comparisons yielded values ranging from 0.0029 to 0.1517. The mean intrapopulation genetic variation was high (0.0993), but limited genetic variation was detected among isolates sampled on each occasion. Taken together this suggests a population comprised of a variety of apparently distantly related clones (genomic groups), each adapted to local conditions. Genome sizes were estimated from the sum of *SpeI* restriction fragments and ranged from 4.2 to 5.5 Mbp. Examination of the distribution of *XbaI*, *SpeI*, *SwaI* and *PacI* restriction endonuclease sites showed that the distribution of *SpeI* sites differed significantly from the expected (random) distribution.

Keywords: fluorescent pseudomonads, phyllosphere, population analysis, systematics, environmental microbiology

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Abbreviations: FAME, fatty acid methyl ester; PFGE, pulsed-field gel electrophoresis; RFLP, restriction fragment length polymorphism; UPGMA, unweighted pair-group method with arithmetic averages.

INTRODUCTION

Fluorescent pseudomonads are prodigious colonizers of plants and occur in large numbers on leaf surfaces (the phyllosphere; Van Outryve *et al.*, 1989; Ercolani, 1991;

Hirano & Upper, 1993; Thompson *et al.*, 1993a), roots, and in the soil immediately adjacent to the root where microbial activity is affected by the plant (the rhizosphere; O'Sullivan & O'Gara, 1992). The intimate nature of the association between pseudomonad and plant is such that bacterial activity can markedly affect plant growth. Certain pseudomonads, including *Pseudomonas syringae* and *P. viridiflava*, can cause disease (Palleroni, 1984), while others, such as isolates of *P. fluorescens* and *P. putida*, can promote plant growth (O'Sullivan & O'Gara, 1992). The majority of phyllosphere-inhabiting fluorescent pseudomonads have no apparent effect on plant growth, but nevertheless represent important components of the leaf community (Austin *et al.*, 1978; Ercolani, 1978; Van Outryve *et al.*, 1989).

Fluorescent pseudomonads represent the largest group of bacteria inhabiting the phyllosphere of field-grown sugar beet. A recent survey based on fatty acid methyl ester (FAME) analysis showed that *P. aureofaciens* (*P. chlororaphis*) was the most frequently isolated species and, significantly, it was the only species isolated on every sampling occasion throughout the season (Thompson *et al.*, 1993a). On one particular occasion *P. aureofaciens* represented up to 83% of the identified bacterial community (Thompson *et al.*, 1993a). The persistence of *P. aureofaciens* and other closely related fluorescent pseudomonads within the phyllosphere is of interest because of the adaptive traits necessary for survival in the face of frequent and often extreme alterations in temperature, moisture content, UV-irradiation and nutrient status (Dickinson, 1986). The precise nature of these traits and adaptive strategies which enhance survival of bacteria in environments subject to frequent and unpredictable change are of considerable interest (Lindow, 1993; Rainey *et al.*, 1993a; Moxon *et al.*, 1994).

The persistence of fluorescent pseudomonads in the phyllosphere renders them suitable and desirable candidates for biocontrol programmes targeted at agricultural crops. Phyllosphere-adapted saprophytes may provide plant protection by excluding pathogens from the plant environment; a direct result of their ability to rapidly and aggressively colonize available niches (Wilson & Lindow, 1993). They may also exclude pathogens by the production of antimicrobial compounds (O'Sullivan & O'Gara, 1992). In addition, and as a result of the ease with which pseudomonads can be genetically manipulated, such strains are potential hosts for foreign genes targeted at the phyllosphere. However, before embarking upon biocontrol projects involving environmental release an understanding of the structure and diversity of the fluorescent pseudomonad population is essential. In particular, it is necessary to determine whether the apparent adaptability inherent within this species is the result of marked phenotypic plasticity or genetic diversity, or a combination of both.

Studies on the *P. syringae* pv. *savastanoi* population of olive leaves showed that specific phenotypes were found on different sampling occasions, suggesting that long-term

survival is dependent upon phenotypic heterogeneity (Ercolani, 1983, 1985); the extent of genotypic diversity among these isolates was not investigated. Population genetic studies based on allozyme analysis indicate that genetic diversity is correlated with habitat variability (see for example, Denny *et al.*, 1988; McArthur *et al.*, 1988), but whether this is true for all phyllosphere inhabitants remains to be determined.

We have attempted in this study to examine the degree of phenotypic and genotypic diversity within a selection of closely related isolates sampled from one field during the course of a single season. During the 1990 field season, 150 randomly selected bacteria were isolated from three leaf types (immature, mature and senescent) on each of seven sampling occasions and subjected to FAME analysis. Cluster analysis of FAME profiles revealed many different taxonomic groups (Thompson *et al.*, 1993a) one of which, the so-named *P. aureofaciens* cluster, formed the basis of this study.

METHODS

Isolation of bacteria. Leaf samples were taken from a sugar beet field (*Beta vulgaris*, var. Amethyst) contained in a 15 m × 15 m plot at the University Field Station, Wytham, Oxford, UK. Bacteria were sampled on seven different occasions throughout the 1990 growing season: 52 d (June), 79 d (July), 114 d (August), 148 d (September), 183 d (November), 220 d (December) and 272 d (January) after sowing. Methods of sampling have been described in detail (Thompson *et al.*, 1993a). Briefly, bacteria were isolated from three replicate samples, each of which was divided into three leaf types (immature, mature, and senescent) and each of which represented a pool of leaves collected from three randomly selected plants (plants sampled on day 52 were too young to be differentiated on the basis of leaf type). Each strain was assigned an isolate number which also indicated the replicate from which it was isolated: isolates 1–50 were isolated from replicate one, isolates 51–100 were isolated from replicate two and isolates 101–150 were isolated from replicate three. The only exception to this scheme were samples collected on day 79, when 60 isolates were taken from replicate three (isolates 101–160). Each isolate number was prefixed by a sample number indicating the day of isolation, for example, 148/128 was isolated on day 148 from replicate three; similarly, strain 52/76 was isolated on day 52 from replicate two. Of the bacteria identified on each sampling occasion, only those identified in the first instance on the basis of FAME profile as *P. aureofaciens* were selected for further study (122 isolates). Of these, a random sample of 30 isolates was chosen for detailed analysis. Once isolated, all cultures were stored at –80 °C and analysed together at the completion of sampling.

Isolation media, culture and storage conditions were as described previously (Thompson *et al.*, 1993a).

Biochemical and carbon source utilization characteristics.

Biochemical characteristics of each isolate were assessed using the API20NE standardized micromethod (bioMérieux), which included eight conventional and 12 assimilation tests. Strips were inoculated according to the manufacturer's instructions and incubated for 24 h at 25 °C before recording the results. Isolate identification was achieved by comparing the reactions recorded for each isolate with the analytical profile index provided by the manufacturer.

The ability of isolates to assimilate a range of sole carbon sources was assessed by the auxanographic API system containing 49 sugars (API50CH), 49 amino compounds (API50AA) and 49 organic acids (API50AO). Isolates were grown overnight on Tryptic Soy Broth (TSB) agar at 28 °C and a suspension of cells (0.04 g wet weight in 60 ml assimilation medium API50LRA) was used to inoculate each cupule. Strips were incubated at 20 °C and growth was recorded (slight, moderate or strong) after 1, 2, 4, 8 and 16 d.

The ability to assimilate each carbon source was treated as a separate unit character. Codes from 0 (negative) to 10 (strongly positive after 1 d incubation), corresponding to the rate and strength of growth, were assigned to each isolate. These data were then subjected to numerical analysis. Similarities between isolates were calculated with the generalized similarity coefficient and the coefficient based on the Euclidean distance between pairs of isolates. This was achieved using UNISTAT software (UNISTAT, Highgate, London, UK) and isolates were clustered using the unweighted pair group method with arithmetic averages (UPGMA) (Sneath & Sokal, 1973).

Cellular fatty acid analysis. Isolates for FAME analysis were cultured on TSB agar plates. Cells were harvested after 24 h incubation at 28 °C and whole-cell fatty acids were saponified, methylated and extracted as described by Miller & Berger (1985). FAME analysis was performed using a Hewlett-Packard model 5890 series II gas chromatograph. All analyses were conducted in triplicate and the mean FAME profiles were used to generate a unique library for each isolate. Fatty acid peaks were named by the Microbial Identification System (MIS) software (Microbial ID, Newark, DE, USA) and isolates identified using the MIS 'Aerobe Library'. Initially all isolates were identified using 'Aerobe Library' version 3.30 (October 1990 update) and subsequently using version 3.50 (October 1991 update). Quantitative data obtained from FAME analysis were subjected to numerical analysis as described previously (Thompson *et al.*, 1993b). Similarities between isolates were calculated using the generalized similarity coefficient of Gower (Gower, 1966) and the coefficient based on the Euclidean distance between pairs of isolates (Bøe & Gjerde, 1980). Isolate clustering was accomplished using the UPGMA.

Pyrolysis mass spectrometry. The organic pyrolysate contents from triplicate overnight cultures of isolates grown on TSB agar at 28 °C were analysed using a Horizon Instruments PYMS 200X pyrolysis mass spectrometer, as described by Orr *et al.* (1991). MS-pyrolysis data were subjected to principal com-

ponent and canonical variate analysis using GENSTAT (Goodacre & Berkely, 1990). Data were transformed to a percentage similarity matrix using Gower's similarity coefficient and isolates were clustered using the UPGMA.

Total cellular protein content. Total cellular protein was prepared from 0.5 ml cells removed from an overnight Luria broth (LB) culture using the method of Xu & Gross (1988) as modified by Rainey *et al.* (1993b). Proteins were separated on both 6% and 10% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS-PAGE) and stained with Coomassie Brilliant Blue (Laemmli, 1970). Gels were photographed and digitized using a Summa graphics tablet interfaced with Molmatch software (UV Products, Cambridge, UK). Only proteins resolved on 6% gels were used in subsequent analysis and a measure of similarity between lanes was obtained using the Dice coefficient, S_d (see below). Isolates were clustered using the UPGMA.

Pulsed-field gel electrophoresis

DNA preparation. Unsheared DNA was prepared by embedding whole cells in chromosomal-grade agarose blocks followed by lysis with detergent and proteinase K (Rainey *et al.*, 1993b). One volume of cells grown overnight in LB was harvested, washed twice in NET-100 buffer [0.1 M NaCl, 0.1 M EDTA, 0.01 M Tris/HCl (pH 8.0)], then mixed with an equal volume of molten 0.9% chromosomal-grade agarose (in NET-100) and dispensed into moulds. Cells were lysed by immersing the agarose blocks in lysis solution I [6 mM Tris/HCl (pH 7.6), 1 M NaCl, 100 mM EDTA (pH 8.0), 0.5% sarkosyl, 1 mg lysozyme ml⁻¹] for 24 h at 37 °C. Lysis solution I was then replaced with solution II [0.5 M EDTA (pH 9.0), 1% sarkosyl, 1.5 mg proteinase K ml⁻¹] and incubation continued for a further 48 h at 50 °C.

Restriction endonuclease digestion. Restriction endonuclease digestions were conducted as described by Suwanto & Kaplan (1989), following PMSF treatment. Restriction endonuclease digestions were performed in 1 × KGB buffer (McClelland *et al.*, 1988) using 8 U enzyme. Reactions containing *Pacl* were incubated at 25 °C for 6 h; reactions containing *XbaI*, *SpeI* and *SwaI* were also incubated for 6 h, but at 37 °C.

Pulsed-field gel electrophoresis. DNA inserts containing approximately 2 µg DNA were loaded into 0.5 × TBE-buffered gels. Gels were run in a CHEF DRII pulsed-field electrophoresis system (Bio-Rad) containing 0.5 × TBE buffer at 200 V and stained in ethidium bromide (0.5 mg ml⁻¹) for 30 min. Fragment

Table 1. Biochemical characteristics of isolates

Biochemical character	Isolate						
	183/147, 114/97, 183/143, 183/112, 183/122, 183/116, 114/74, 114/60, 114/82, 114/80, 114/110, 148/128, 183/145, 148/124	79/117, 272/48	114/85, 114/95, 148/146	148/102, 114/147	79/45, 79/13, 79/2	52/76	79/156, 79/123, 79/146, 79/148, 79/61
Nitrate reduction	+	+	+	+	—	+	+
Arginine dihydrolase	+	+	+	+	—	+	+
Urease production	—	+	+	+	—	+	—
Aesculin hydrolysis	—	—	—	—	+	+	—
Gelatin hydrolysis	+	—	+	—	+	—	—
Oxidase	+	+	+	+	—	+	+
Maltose	—	—	—	+	—	—	—

Table 2. Sole carbon source assimilation characteristics of isolates from clusters/subclusters defined by UPGMA analysis using Euclidean distance

The percentage frequency of features present within each cluster/subcluster is shown. Carbon sources universally utilized or not used by any isolate are given in footnotes.

Cluster ...		A			B			
Subcluster ...		A1	A2	A3		B1	B2	B4
Source* ...		I	I	I/M		S/U	I/M	S
No. of isolates ...	19†	5	4	8	11†	2	5	3
Growth in API 50 AO on:								
Butyrate	95	100	100	100	82	100	100	33
n-Valerate	100	100	100	100	91	100	100	67
Isovalerate	100	100	100	100	0	0	0	0
Malonate	95	80	100	100	100	100	100	100
Maleate	85	80	71	75	100	100	100	100
Glutarate	90	100	100	100	100	100	100	100
Sebacate	20	0	43	38	0	0	0	0
D-Malate	95	100	85	88	100	100	100	100
D-Tartrate	25	80	100	13	0	0	0	0
L-Tartrate	50	0	100	100	0	0	0	0
meso-Tartrate	100	100	100	100	36	0	20	100
Laevulinate	95	100	100	100	63	100	100	0
2-Oxoglutarate	95	100	86	88	100	100	100	100
Citraconate	75	100	0	100	100	100	100	100
Mesaconate	5	0	0	13	36	100	40	0
Phenylacetate	15	40	0	0	0	0	0	0
Benzoate	95	100	100	100	9	50	0	0
3-Hydroxybenzoate	5	0	0	0	0	0	0	0
4-Hydroxybenzoate	95	100	100	100	100	100	100	100
D-Mandelate	5	0	0	0	0	0	0	0
L-Mandelate	5	0	0	0	9	0	20	0
Phthalate	5	0	0	0	0	0	0	0
Isophthalate	5	0	0	0	0	0	0	0
Terephthalate	5	0	0	0	9	50	0	0
Growth in API 50 CH on:								
Erythritol	5	0	0	0	91	100	100	67
D-Arabinose	11	20	0	0	18	0	40	0
L-Arabinose	94	80	100	100	100	100	100	100
D-Xylose	89	60	100	100	100	100	100	100
Adonitol	5	0	0	13	91	100	100	67
Methyl β -D-xyloside	0	0	0	0	45	0	83	0
Sorbose	5	0	25	0	0	0	0	0
Rhamnose	0	0	0	0	73	100	50	100
Inositol	0	0	0	0	100	100	100	100
Sorbitol	0	0	0	0	100	100	100	100
Methyl α -D-glucoside	0	0	0	0	9	0	17	0
Amygdalin	5	0	0	0	9	0	20	0
Arbutin	0	0	0	0	91	50	100	100
Aesculin	100	100	100	100	91	100	83	100
Cellobiose	5	0	0	13	9	0	17	0
Maltose	17	0	25	25	36	0	40	67
Melibiose	5	0	0	13	18	0	40	0
Inulin	11	0	25	13	0	0	0	0
Raffinose	11	0	0	13	91	50	100	100
Starch	39	0	50	63	27	0	20	67
Xylitol	0	0	0	0	100	100	100	100
Gentiobiose	0	0	0	0	9	0	20	0

Table 2 (cont.)

Cluster...		A				B			
Subcluster...		A1	A2	A3		B1	B2	B4	
Source*...		I	I	I/M		S/U	I/M	S	
No. of isolates...	19†	5	4	8	11†	2	5	3	
D-Turanose	5	0	0	13	9	0	20	0	
D-Lyxose	83	60	100	88	100	100	100	100	
D-Tagatose	5	0	0	13	9	0	20	0	
D-Fucose	5	0	0	0	9	50	0	0	
L-Fucose	11	0	0	13	18	0	0	67	
L-Arabitol	5	0	0	13	100	100	100	100	
5-Ketogluconate	17	20	0	13	100	100	100	100	
Growth in API 50 AA on:									
Glycine	90	100	100	88	73	100	67	67	
L-Norleucine	85	100	100	75	73	100	83	33	
DL-Norvaline	75	100	100	75	82	100	100	67	
DL-2-Aminobutyrate	10	0	0	13	27	0	40	33	
L-Threonine	75	60	75	88	100	100	100	100	
L-Cysteine	0	0	0	0	73	50	33	33	
L-Methionine	0	0	0	0	91	100	83	100	
L-Histidine	95	100	100	100	100	100	100	100	
D-Tryptophan	0	0	0	0	9	0	20	0	
L-Tryptophan	9	0	0	13	100	100	100	100	
Trigonelline	100	100	100	100	91	100	67	67	
L-Ornithine	95	100	100	100	100	100	100	100	
L-Lysine	95	100	100	100	100	100	100	100	
L-Citrulline	75	100	75	63	100	100	100	100	
DL-Kynurenine	0	0	0	0	91	50	100	100	
Creatine	0	0	0	0	45	50	50	33	
DL-3-Aminobutyrate	25	40	50	13	55	100	67	0	
DL-5-Aminovalerate	95	100	100	100	95	100	100	100	
2-Aminobenzoate	0	0	0	0	73	100	100	0	
Acetamide	10	40	0	0	19	0	0	67	
Amylamine	0	0	0	0	54	0	83	33	
Spermine	100	100	100	100	27	100	0	33	
Histamine	20	0	100	0	0	0	0	0	
Tryptamine	0	0	0	0	91	50	100	100	

*I, immature leaves; M, mature leaves; S, senescent leaves; U, undifferentiated plant leaves.

† Including single isolates.

Compounds utilized by all isolates as sole carbon sources: acetate, propionate, isobutyrate, n-caproate, heptanoate, caprylate, pelargonate, caprate, succinate, fumarate, DL-lactate, DL-glycerate, DL-3-hydroxybutyrate, L-malate, pyruvate, aconitate, citrate, D-alanine, L-alanine, L-leucine, L-isoleucine, L-valine, L-serine, L-phenylalanine, L-tyrosine, L-aspartate, L-glutamate, L-arginine, L-proline, betaine, β -alanine, DL-4-aminobutyrate, sarcosine, ethanolamine, diaminobutane, glucosamine, glycerol, ribose, galactose, glucose, fructose, mannose, mannitol, N-acetylglucosamine, sucrose, trehalose, D-arabitol, gluconate, 2-ketogluconate.

Compounds not utilized as sole carbon sources by any isolates: oxalate, adipate, pimelate, suberate, azelate, glycolate, itaconate, 2-hydroxybenzoate, 3-aminobenzoate, 5-aminobenzoate, urea, ethylamine, butylamine, benzylamine, L-xylose, dulcitol, methyl-D-mannose, salicin, lactose, melezitose, glycogen.

sizes were estimated by comparison with comigrating ladders of lambda DNA concatemers (Bio-Rad) and *EcoRI*/*HindIII*-digested lambda DNA; *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were also used as molecular mass standards.

Evaluation of PFGE gels. Enlarged prints of gels were digitized using a Summagraphics data tablet and fragment sizes determined using Molmatch computer software (UV Products).

Criteria for evaluating gels were as described by Grothues & Tümmeler (1991); a minimum of two gels were run per enzyme digest to ensure optimal resolution of all fragments.

Relatedness of isolates was determined by conducting comprehensive pair-wise comparisons of macrorestriction fragment sizes using the Dice coefficient, S_D , which is defined as the ratio of twice the number of bands common to each pair of restriction

profiles to the total number of macrorestriction fragments in each restriction profile (Nei & Li, 1979).

$$S_D = 2n_i/(n_A + m_B)$$

where n_A and m_B are the number of fragments in patterns A and B, respectively, and n_i is the number of common bands. For each pair of isolates S_D values were determined from the number of common bands and total number of bands generated by all four enzymes. The statistical significance of pair-wise comparisons was evaluated using equation 13 of Grothues & Tümmeler (1991), which indicates the confidence interval for the statistical *a priori* similarity of macrorestriction fragment profiles:

$$P(S_{D,AB}) = 2/(\sum_K n_K + \sum_K m_K) \times [\sum_K (n_K m_K / I_K) \pm t/2(\sum_K n_K)^{1/2}]$$

where K indicates the number of restriction profiles generated by K independent enzyme digestions, I is the number of intervals into which the range of restriction fragments is divided and t is the value of Student's t -distribution (see Grothues & Tümmeler, 1991). For both *Xba*I and *Spe*I digests, each range of restriction fragments was divided into the following intervals: 0.5 kbp between 0 and 15 kbp; 1 kbp between 15 and 24 kbp; 2 kbp between 25 and 50 kbp; 3 kbp between 51 and 99 kbp; 5 kbp between 100 and 200 kbp; and 10 kbp above 200 kbp. Intervals for both *Pac*I and *Swa*I macrorestriction profiles were: 2 kbp between 4 and 52 kbp; 5 kbp between 53 and 198 kbp; and 10 kbp above 200 kbp.

Results from this analysis were interpreted as described by Grothues & Tümmeler (1991). Briefly, an S_D value within the calculated confidence interval (for example 95%) indicated that the level of similarity between a pair of isolates was no more than that expected by chance, given a random distribution of restriction sites and a given genome size. Such a result indicated that the isolates compared belong to the same species (isolates belonging to the same species are considered to have similar DNA base compositions, codon usage characteristics and genome sizes). An S_D value outside the 95% confidence interval indicated that the isolates are either significantly more closely related (if the S_D was higher) or less closely related (if the S_D was lower) ($P < 0.05$) than expected on the basis of genome sizes and a random distribution of restriction sites. If the S_D value was greater than the upper value of the 95% confidence interval, then the isolates compared probably share a common ancestor (and may be assigned to the same biovar). If the S_D value was less than the lower value of the 95% confidence interval, then the isolates compared probably do not belong to the same taxospecies (Grothues & Tümmeler, 1991).

Genetic variation as determined by macrorestriction fragment analysis was also expressed in terms of nucleotide diversity (π), where the average number of nucleotide differences between isolates was calculated using the equation

$$\pi = [(-\ln S_D)/r]$$

where r is the number of nucleotide base pairs in the restriction endonuclease recognition site (Nei & Li, 1979).

RESULTS

Biochemical and carbon source utilization characteristics

Results from the standardized micromethod API20NE tests showed that all isolates were positive for catalase production and assimilation of arabinose, caprate, citrate, gluconate, glucose, malate, mannitol, mannose and *N*-

acetylglucosamine, but negative for β -galactosidase and indole production, acidification of glucose, reduction of nitrate to dinitrogen gas, and assimilation of adipate and phenylacetate. The seven variable reactions are listed in Table 1. On the basis of these results and using the API analytical profile index, all isolates were identified as *P. fluorescens*, the exceptions being isolates 79/2, 79/13, 79/45, 79/117, 114/95, 114/85, 148/146 and 272/48, which were identified to the genus *Pseudomonas*. The API profile index does not contain a wide selection of *Pseudomonas* strains and therefore the results of the API20NE tests were also compared with the determinative schemes presented by Palleroni (1984). On the basis of these schemes all isolates were identified as *P. fluorescens*, the exceptions being isolates 79/2, 79/13 and 79/45 which were identified as *P. syringae*. This distinction was made primarily on the basis of the results of the arginine dihydrolase and oxidase tests.

Of the 147 auxanographic tests contained on the API strips API50CH, API50AA and API50AO, 49 carbon sources were utilized by all isolates and 21 were not utilized by any isolate (Table 2). Carbon sources which showed variable utilization are listed in Table 2. The auxanographic API system was found to be highly reproducible and duplicate strips of two randomly selected isolates were 97.4% similar.

Numerical analysis revealed two major clusters (Fig. 1). Isolates taken from the phyllosphere on days 114, 148 and 183 were grouped into cluster A, while isolates sampled on days 52, 79 and 272 were grouped into cluster B. At a Euclidean distance of 22, isolates in cluster A were grouped into five subclusters, and with the exception of isolate 183/145, isolates subclustered according to time of isolation and to a lesser extent according to leaf type. Subcluster A1 contained isolates sampled on day 148 from immature leaves plus isolate 183/145, which was also taken from immature leaves 35 d later. Subcluster A2 contained isolates sampled on day 183 from immature leaves, subcluster A3 contained isolates sampled on day 114 from both immature and mature leaves, and subclusters A4 and A5 each contained a single isolate isolated on sampling day 183 and 114, respectively. Subdivision of cluster B at 22 Euclids yielded four subclusters, of which B2, B3 and B4 contained isolates sampled on day 79. Subcluster B4 contained isolates from senescing leaves, while B2 contained a mixture of isolates from mature and immature leaves. Subcluster B1 contained two isolates sampled 220 d apart: one from undifferentiated plants on day 52 and the other from senescing leaves on day 272.

A number of carbon sources proved useful in distinguishing between clusters A and B, and included: adonitol, arbutin, benzoate, L-cysteine, erythritol, inositol, *iso*-valerate, DL-kynurenine, L-methionine, rhamnose, sorbitol, tryptamine, L-tartrate and xylitol. Carbon sources facilitating subdivision of cluster A included: L-arabitol, histamine, sebacate, starch, D-tartrate, L-tartrate and *meso*-tartrate. Carbon sources facilitating subdivision of cluster B included: 2-aminobenzoate, DL-3-

Fig. 1. Dendrogram representing the relationship between 30 fluorescent *Pseudomonas* isolates based on the ability to assimilate 147 sole carbon sources. The origin of isolates is indicated as follows: U, leaves from plants < 53 d old; I, immature leaves; M, mature leaves; S, senescent leaves.

aminobutyrate, laevulinate, mesaconate, spermine, *meso*-tartrate and terephthalate (Table 2).

Comparison of the carbon source assimilation profiles with the diagnostic tables provided by Palleroni (1984) confirmed the previously assigned species designations, but also enabled identification of isolates to biovar/pathovar level. Of the 84 variable characteristics listed in Table 4.9 by Palleroni (1984), 64 were included amongst the auxanographic tests. Isolates in cluster A bore greatest similarity to biovar V of *P. fluorescens*; isolates in subcluster A1 differed in their ability to assimilate acetamide, D-alanine and citraconate, and isolates in subclusters A2 and A3 differed in their ability to assimilate D-alanine and L-tartrate. Isolates in subclusters B1 and B2 bore greatest similarity to biovar III of *P. fluorescens* and differed only in their ability to assimilate sucrose.

Isolates in subcluster B4 bore greatest similarity to *P. syringae* and were distinguished from *P. viridiflava* primarily on the basis of raffinose and D-tartrate assimilation; however, the ability of these isolates to assimilate L-leucine, L-tryptophan and L-tyrosine rendered them slightly atypical (Palleroni, 1984). From a total of 28 nutritional characteristics listed in Table 4.13 by Palleroni (1984) for differentiating *P. syringae* pathovars, *P. viridiflava* and *P. cichorii*, 19 were included in the current study. The

assimilation pattern of isolates in subcluster B4 bore greatest similarity to *P. syringae* pv. *syringae*, with the only difference residing in the ability to assimilate propionate.

Carbon source assimilation data also indicated that the original species designation of *P. aureofaciens*, given by the MIDI library (version 3.30, Oct. 1990), was incorrect. All isolates differed from *P. aureofaciens* on the basis of D-alanine, citraconate, glycine, D-malate, phenylacetate, trigonelline and D-xylose assimilation; furthermore, no isolate secreted the distinctive orange phenazine pigment typical of *P. aureofaciens* (Palleroni, 1984; Johnson & Palleroni, 1989).

Cellular fatty acid analysis

Under standardized growth conditions all isolates displayed qualitatively similar FAME profiles. Two fatty acids, *cis*-9 hexadecenoic ($C_{16:1}$ *cis*) acid and hexadecanoic ($C_{16:0}$) acid, together represented over 60% of the total fatty acid content of each isolate. The remaining fatty acid content (representing > 1% of the total) was composed of 3-hydroxydecanoic ($C_{10:0}$ 3OH), dodecanoic ($C_{12:0}$), 2-hydroxydodecanoic ($C_{12:0}$ 2OH), 3-hydroxydodecanoic ($C_{12:0}$ 3OH) and 9,10-methylenehexadecanoic acid ($C_{17:0}$ *cyclo*) and an isomer of octadecenoic acid ($C_{18:1}$).

Fig. 2. Dendrogram representing the relationship between 30 fluorescent *Pseudomonas* isolates based on FAME analysis. The origin of isolates is indicated as in Fig. 1.

Results from triplicate fatty acid determinations were highly reproducible, both quantitatively and qualitatively. The coefficient of variation [(standard deviation/mean) $\times 100$] for triplicate samples of each fatty acid representing $> 4.1\%$ of the total content was $< 7.1\%$. Individual fatty acid profiles were compared with entries from two MIS 'Aerobe' libraries: Rev. 3.3 – Oct. 1990 and Rev. 3.5 – Oct. 1991. In the initial analysis all isolates exhibited profiles closest to that of *P. aureofaciens*, but when the updated 'Aerobe' library was used (Rev. 3.5), isolates 79/2, 79/13 and 79/45 were identified as *P. s. syringae* (with similarity indices of between 0.79 and 0.87); the remainder were identified as *P. aureofaciens* (with similarity indices ranging from 0.83 to 0.94), although in terms of general phenotypic properties these isolates bore closer resemblance to *P. fluorescens* (see above).

Numerical analysis revealed two main clusters (A and B) (Fig. 2) which were identical in composition to clusters A and B obtained on the basis of sole carbon source assimilation data (Fig. 1), but the arrangement of isolates within these clusters differed. With the exception of two cases (isolates 114/95 and 79/61) all isolates clustered according to leaf type. Within cluster A five subclusters were delineated at 2 Euclids and these showed many similarities with carbon source assimilation clusters. In general subcluster A1 corresponded to carbon source

assimilation subcluster A2, subcluster A3 corresponded to carbon source assimilation subcluster A3 and subcluster A5 corresponded to carbon source assimilation subcluster A1. Within cluster B four subclusters were delineated at 2 Euclids. Isolates 272/48 and 52/76 no longer clustered together, but isolates sampled on day 79 were subclustered into two distinct groups: B2 and B4.

Fatty acids which most distinguished isolates in cluster A from those in cluster B were $C_{12:0}$ and $C_{12:0} 2OH$. In most cases the differences in individual fatty acid contents of isolates from adjacent subclusters were small (ratio < 0.5), but when these differences were combined, good separation between subclusters was achieved.

Pyrolysis mass spectrometry

Numerical analysis of the MS-pyrolysis data (Fig. 3) revealed a relationship between isolates similar to that indicated by numerical analysis of carbon source assimilation data and FAME data (Figs 1 and 2). MS-pyrolysis delineated isolates into two clusters (A and B) and these were identical in composition to the clusters based upon carbon source assimilation and FAME analysis. With the exception of isolate 148/102, isolates sampled on days 148 and 183 from immature leaves subclustered together. Isolates sampled on day 114 were

Fig. 3. Dendrograms representing the relationship between 30 fluorescent *Pseudomonas* isolates based on MS-pyrolysis. Isolates in clusters A and B were analysed separately to achieve better subcluster resolution. The origin of isolates is indicated as in Fig. 1.

distributed throughout four subclusters and were not clustered according to leaf type. Cluster B was subdivided into three subclusters, with isolates sampled on day 79 being divided between two subclusters. Isolates 272/48 and 52/76 clustered together.

Total soluble protein content

One-dimensional SDS-PAGE of whole-cell protein extracts from the 30 isolates on 6% polyacrylamide gels generated between 36 and 46 bands which ranged in size from approximately 400 kDa to 40 kDa (bands smaller than 40 kDa were not used in the analysis). Duplicate gels with the same molecular mass markers yielded the same size bands ($\pm 2\%$), indicating the reproducibility of the method.

Numerical analysis revealed six clusters at the 90% level of similarity (Fig. 4). In general, isolates were clustered according to day of sampling, but isolate 183/145 clustered with isolates sampled on day 148, and isolates 52/76 and 272/48 clustered together. With few exceptions, members of a cluster shared identical protein

Fig. 4. Dendrogram representing the relationship between 30 fluorescent *Pseudomonas* isolates based on total cellular proteins resolved by 6% SDS-PAGE. The origin of isolates is indicated as in Fig. 1.

profiles. Isolates 114/60, 114/82 and 114/147 possessed identical profiles to the other isolates sampled on day 114, but isolates 114/60 and 114/82 possessed one extra band and isolate 114/147 lacked five high molecular mass bands (Fig. 5). The high molecular mass protein bands were especially useful for discriminating between isolates.

Quantitative evaluation of macrorestriction patterns

Four rare-cutting restriction endonucleases, *PacI* (5'-TT/AATTA), *SpeI* (5'-A/CTAGT), *SwaI* (5'-ATTT/AAAT) and *XbaI* (5'-T/CTAGA), were used to cleave the G+C-rich *Pseudomonas* genome and yielded between 8 and 11; 32 and 47; 12 and 17; and 39 and 57 fragments, respectively (exclusive of doublets/triplets). The value of the extremely rare-cutting enzymes, *PacI* and *SwaI*, was limited because of the small number of bands. Nevertheless, while S_D values for *PacI* and *SwaI* were lower than for *XbaI* and *SpeI*, the effect of these enzymes on clustering and subsequent statistical analysis was negligible. It is important to recognize the effect that different enzymes have on the analysis of macrorestriction fragments (Giovannetti *et al.*, 1990). The most useful data were obtained from *XbaI* and *SpeI*, which generated approximately 35 and 45 bands, respectively, and almost all bands were clearly resolved by PFGE. Enzymes which cleaved either more, or less, frequently resulted in either an overestimate, or underestimate, respectively, of the degree of similarity.

Fig. 5. SDS-PAGE (6% polyacrylamide) of total cellular protein from *Pseudomonas* isolates. (a) Electrophoretic protein patterns of isolates sampled on sampling days 52, 79 and 272. (b) Electrophoretic protein patterns of isolates sampled on sampling day 114 (and repeat of 52/76 and 272/48). (c) Electrophoretic protein patterns of isolates sampled on sampling days 148 and 183. Molecular mass markers are indicated on the right.

PFGE revealed the presence of eight distinct genomic (clonal) groups designated I, II, III, IIIP, IV, V, VI, and VII (Fig. 6, Table 3), and in general, isolates sampled on the same day, from identical leaf types, were members of

Table 3. Division of fluorescent *Pseudomonas* isolates into genomic groups on the basis of the macrorestriction fragment analysis and approximate genome size

Isolates	Genomic group	Genome size* (Mbp)
79/2, 79/13, 79/45	I	4.2
79/61, 79/117, 79/123, 79/146, 79/148, 79/156	II	4.8
114/74, 114/80, 114/82, 114/85, 114/95, 114/97, 114/110, 114/147	III	4.9
114/60	IIIP	5.3
148/102, 148/124, 148/128, 148/146, 183/145	IV	5.5
183/112, 183/116, 183/122, 183/143, 183/147	V	5.3
52/76	VI	4.7
272/48	VII	4.7

* Estimate of genome size is based upon summation of *SpeI* macrorestriction fragments.

the same genomic group. Two exceptions to this trend were isolates sampled on day 114, where genomically identical bacteria were isolated from different leaf types, and isolates sampled on day 183, where isolate 183/145 possessed the same macrorestriction profile as isolates belonging to genomic group IV (isolates sampled on day 148). Isolate 114/60 is a variant of genomic group III, designated IIIP. The restriction profile of this isolate showed an additional one, or two, bands depending upon the enzyme with which the DNA was cleaved. Examination of these additional bands showed that they fluoresced with less intensity when compared to similar-sized bands from the same genomic digest and it is likely that they are plasmid DNA. On the basis of band sizes from *PacI*, *SpeI* and *SmaI* digests, the plasmid was estimated to be 400 kbp. Attempts to isolate the plasmid using the method described by Wheatcroft & Williams (1981) proved unsuccessful, although similar-sized plasmids were isolated from control bacteria (A. K. Lilley, unpublished data). Absence of this plasmid from the other eight members of genomic group III suggests that it may have been acquired as the result of a recent *in situ* transfer event. The presence of the plasmid did not result in any detectable phenotypic differences.

Approximate genome sizes were obtained by summation of *SpeI* restriction fragments and ranged from 4.2 Mbp to 5.5 Mbp; *P. s. syringae* isolates had the lowest genome size.

Distribution of restriction fragments. The distribution of restriction fragments resulting from digestion of a member of each genomic group with each of the four

Fig. 6. Separation of *Xba*I (a), *Spe*I (b), *Pae*I (c) and *Sma*I (d) digests of fluorescent *Pseudomonas* chromosomes representative of each genomic group by contour-clamped homogeneous electric field gel electrophoresis. Representative isolates and corresponding genomic groups are: isolate 79/45, genomic group I; isolate 79/61, genomic group II; isolate 114/110, genomic group III; isolate 114/60, genomic group IIIP; isolates 148/128 and 183/145, genomic group IV; isolate 183/143, genomic group V; isolate 52/76, genomic group IV; isolate 272/48, genomic group VII. Fragments were resolved by using a ramped pulsed time of: 1–20 s for 22 h (1.25% agarose) (a); 1–25 s for 22 h (1.25% agarose) (b); 30–75 s for 21 h (1.0% agarose) (c); 30–75 s for 21 h (1.05% agarose) (d). The sizes of comigrating lambda concatemers and *Saccharomyces cerevisiae* (S.c.) chromosomes are given in kbp.

enzymes was plotted alongside the theoretical distribution of fragments calculated using the distribution function

$$P(x) = \mu(1 - \mu)^{x-1}$$

where, assuming a random distribution of enzyme recognition sites, μ is the reciprocal of the mean fragment length, l_m , and x is fragment size (Grothues & Tümmeler, 1991). The result of this analysis is shown in Fig. 7 for *Spe*I, which was the only enzyme that yielded a distribution of fragments which differed significantly from

the expected distribution; $\chi^2_{[40]}$ ($P < 0.01$). Deviations were apparent throughout the entire range, but maxima were detectable approximately every 20–30 kbp.

Similarity of fragment patterns. Similarity of fragment patterns was determined by pair-wise comparisons and quantified using the Dice coefficient (Table 4) (Grothues & Tümmeler, 1991). The conversion formula of Nei & Li (1979) was used to express the data in terms of nucleotide diversity (Table 4). Confidence intervals for the statistical

Table 4. Pair-by-pair comparisons of nucleotide diversity (π) values and Dice coefficients (S_D) for fluorescent pseudomonad isolates

Genomic group	Nucleotide diversity value or Dice similarity coefficient* for genomic group:						
	I	II	III	IIIP	IV	V	VII
I		0.4118	0.4364	0.4356	0.3458	0.3792	0.3860
II	0.1268		0.4561	0.4549	0.4414	0.4292	0.4087
III	0.1185	0.1121		0.9799	0.4622	0.4426	0.4365
IIIP	0.1187	0.1125	0.0029		0.4609	0.4417	0.4358
IV	0.1517	0.1168	0.1103	0.1107		0.3930	0.4310
V	0.1386	0.1208	0.1165	0.1167	0.1334		0.5021
VI	0.1360	0.1091	0.1184	0.1187	0.1203	0.0984	
VII	0.1290	0.1278	0.1074	0.1078	0.1115	0.1233	0.0734

* Values below the diagonal are π values; those above the diagonal are S_D values. Numbers in **bold** type indicate that the restriction fragment patterns are significantly more related than expected by chance ($P < 0.001$). All other pair-wise comparisons of restriction fragment patterns were neither more nor less related than expected by chance, i.e. S_D resided within the 95 % confidence interval.

Fig. 7. Distribution of *SpeI* restriction fragments from eight fluorescent *Pseudomonas* isolates—one from each genomic group. The theoretical distribution of fragments (assuming a random distribution of enzyme recognition sites) is shown by the dotted line.

correspondence of each pair of patterns were calculated as described above and used as a means of discriminating between isolates.

A marked degree of heterogeneity was observed amongst macrorestriction profiles; however, S_D values for all pair-wise comparisons resided within the 95 % confidence interval, indicating that all isolates are members of the same taxospecies (Grothues & Tümmeler, 1991). Only two pairs of macrorestriction profiles were found to be significantly more related than expected by chance. Isolate 114/60 was closely related to isolate 114/80 and the other day 114 isolates belonging to genomic group III ($P < 0.001$), and two isolates sampled 220 d apart, 52/76 and 272/48, shared more than 60 % similarity and were also significantly more related than expected by chance ($P < 0.001$).

Numerical analysis was used to group the isolates according to the percentage similarity of macrorestriction fragments as determined using S_D . Groups were clustered using the UPGMA linkage method (Sneath & Sokal, 1973) and the dendrogram shown in Fig. 8 revealed six clusters at, or below, the 60 % level of similarity. With the exception of isolates 52/76 and 272/48 in cluster A, and isolate 183/145 in cluster C, all isolates grouped according to sampling occasion.

No correlation was observed between macrorestriction fragment clusters and the clusters resulting from phenotypic analysis. In fact the diversity in macrorestriction fragment profiles was high, both within and between both biovars and species—there was as much variation in macrorestriction fragment profiles among *P. fluorescens* biovar V isolates as there was between *P. fluorescens* biovar III and *P. fluorescens* biovar V isolates (and indeed the variation within either *P. fluorescens* biovar III or V was as great as that detected between *P. fluorescens* and *P. syringae*).

DISCUSSION

Analysis of both phenotype and genotype has provided a unique perspective on a subgroup of the phyllosphere-inhabiting fluorescent *Pseudomonas* population. Our initial aim was to investigate the extent of phenotypic and genotypic diversity within a sample of bacteria from the phyllosphere of sugar beet which FAME analysis had indicated were closely related. Extensive assessment of phenotypic properties using auxanographic tests, FAME analysis, MS-pyrolysis and total cellular protein profiles confirmed the relatedness of these bacteria, but also revealed internal groupings. In particular, auxanographic tests, cellular fatty acid analysis and MS-pyrolysis showed a division between isolates sampled on sampling days 114, 148 and 183 (cluster A), and those isolated on sampling days 52, 79 and 272 (cluster B). Interestingly, all isolates in cluster A were isolated during the hottest part of the season (sampling days 114, 148 and 183) and produced

Fig. 8. Dendrogram representing the relationship between 30 fluorescent *Pseudomonas* isolates based on the percentage similarity of macrorestriction fragments. The origin of isolates is indicated as in Fig. 1.

copious amounts of exopolysaccharide. Exopolysaccharide production may confer a selective advantage upon phyllosphere bacteria inhabiting leaf surfaces during mid-summer by increasing their resistance to desiccation (Morgan & Beckwith, 1939; Davenport, 1980; Robertson & Firestone, 1992).

In general there was a good correlation between sub-clusters and isolate origin (day of isolation and leaf type). Fatty acid analysis showed a propensity to cluster isolates on the basis of leaf type, whereas auxanographic tests resulted in clustering of isolates according to day of sampling. MS-pyrolysis clustered some isolates according to leaf type, for example isolates sampled from immature leaves on days 148 and 183, but also clustered some isolates according to day of isolation. A correlation between phenotype and habitat or sampling occasion has been observed in other bacterial species. Fatty acid analysis showed that *Xanthomonas maltophilia* isolates sampled early in the season were phenotypically distinct from those sampled later in the season (Thompson *et al.*, 1993b). Similarly, numerical analysis of phenotypic characteristics of *P. s. savastanoi* isolates sampled from olive leaves throughout a season showed that isolates clustered according to their origins (Ercolani, 1983, 1991). Races of *P. s. glycinea* were also shown to differ depending

upon the growth stage of the host (Mew & Kennedy, 1982).

Two methods of phenotypic analysis, namely biochemical tests (combined with carbon source assimilation analysis), and FAME analysis, enabled the isolates to be identified to the species level, or below. On the basis of biochemical tests (and carbon source assimilation data) isolates sampled on days 114, 148 and 183 were identified as *P. fluorescens* biovar V and with the exception of isolates 79/45, 79/13 and 79/2, which were identified as *P. s. syringae*, the remaining isolates were identified as *P. fluorescens* biovar III. These divisions were largely supported by the results of each method of phenotypic analysis, but the distinction between *P. fluorescens* biovar III and biovar V isolates was not clear from the results arising from numerical analysis of total cellular protein profiles. Interestingly, all the phenotypic methods showed that the *P. s. syringae* isolates bore more similarity to *P. fluorescens* biovar III than *P. fluorescens* biovar III isolates showed to *P. fluorescens* biovar V isolates.

The clusters arising from numerical analysis of FAME data bore considerable similarity to the clusters arising from numerical analysis of both carbon source assimilation data and MS-pyrolysis data; however, the species designation *P. aureofaciens*, assigned by the MIS 'Aerobe' library (Rev. 3.5) to all isolates, with the exception of 79/45, 79/13 and 79/12, appears, on the basis of biochemical and carbon source utilization data, to have been incorrect. The identification of isolates 79/45, 79/13 and 79/2 as *P. s. syringae* was in agreement with biochemical and carbon source utilization data. The ability of FAME analysis to differentiate phenotypically similar organisms is impressive, but doubt is cast, at least in some instances, on the validity of the identifications given by the MIS library. The difficulties associated with isolate identification may also reflect the complexity of *Pseudomonas* taxonomy (Palleroni, 1984).

The assignment of isolates to clusters/subclusters on the basis of phenotypic characters was largely supported by the results of the genomic analysis, although with the exception of total soluble protein analysis, members of a genomic group were not always clustered. For example, on the basis of carbon source assimilation data, isolates 183/116, 114/95 and 79/123 did not cluster with members of their respective genomic group. In each instance, the failure to cluster all members of a genomic group was attributable to small, but cumulative differences in the rate of assimilation of carbon sources, rather than to genuine differences in the ability of particular isolates to assimilate particular carbon sources. This result therefore reflects the subjective scoring of these data and was exacerbated by the fact that the isolates within both clusters A and B shared marked phenotypic similarity and thus small differences in assimilation rates had a significant effect on clustering.

Error inherent within the FAME and MS-pyrolysis analytical methods, combined with the very high level of

phenotypic similarity between isolates, may also have accounted for the lack of exact correlation between phenotypic clusters and genomic groups. However, the high correlation between habitat (leaf type) and FAME cluster suggests the possibility that some of these differences may be genuine. That phenotype can vary among members of a genomic group is demonstrated convincingly by the total soluble protein profiles of the genomic group III isolates (isolates sampled on day 114). These phenotypes were heritable and were not lost despite multiple transfers in broth culture and on agar media. Alterations in phenotype which are maintained independently of environmental signals must reflect underlying alterations in the genome (in either DNA sequence or DNA conformation), but these alterations would go undetected unless either a *PacI*, *SpeI*, *SmaI* or *XbaI* enzyme recognition sequence was altered. A significant aspect of the discovery of variable phenotypes within genomic group III is that it provides evidence of intracolonial polymorphism in the natural environment. This adds further credence to a recent hypothesis which argues that production of phenotypic variants within a clonal bacterial population reflects an adaptive strategy which facilitates genetic adaptation of clonal bacterial populations to unpredictable environmental change (Rainey *et al.*, 1993a; Moxon *et al.*, 1994).

Macrorestriction fragment analysis is a powerful method for investigating bacterial population structure and phylogeny (Arbeit *et al.*, 1990; Böhm & Karch, 1992; Harsono *et al.*, 1993; Grothues & Tümmeler, 1991; Rainey *et al.*, 1994b). It overcomes the problems associated with frequently-cleaving restriction endonucleases and unlike RFLP analysis, which detects only a small number of variable sites within the genome, macrorestriction fragment analysis treats the entire genome as a single highly polymorphic RFLP (Grothues & Tümmeler, 1991). The large number of detectable polymorphisms (which can be increased by using more enzymes) combined with unequivocal band resolution enables detection of a variety of alterations in DNA sequence, including mutations in both coding and noncoding regions, inversions or transpositions, and duplications or deletions (Arbeit *et al.*, 1990). Macrorestriction fragment analysis also enabled an examination of the distribution of restriction endonuclease sites. This analysis revealed a non-random distribution of *SpeI* restriction sites within the study group of fluorescent *Pseudomonas* isolates. Non-random distribution of rare palindromes is thought to be important in determining chromosome structure and has been shown in both *P. aeruginosa* (Grothues & Tümmeler, 1991) and *P. stutzeri* (Rainey *et al.*, 1994).

Variation in macrorestriction fragment profiles among the eight genomic groups was unexpectedly pronounced given the high degree of phenotypic similarity among the 30 isolates. While no two macrorestriction fragment profiles were less related than expected by chance (and therefore all isolates could be assigned to the same taxospecies), the highly divergent profiles raise questions relating to *Pseudomonas* taxonomy, the extent of genomic

diversity within the *Pseudomonas* population, and the nature of the evolutionary forces operating to generate and maintain this diversity.

The so-called fluorescent pseudomonads are a notoriously complex and diverse group of bacteria and with the notable exception of *P. aeruginosa*, are highly heterogeneous (Palleroni, 1986). Recent studies using nucleic acids (Palleroni *et al.*, 1972, 1973; Johnson & Palleroni, 1989) have enabled subdivision of the genus into five rRNA homology groups (Palleroni, 1984) and sequence analysis of the 16S rRNA molecule (Woese, 1987) has provided a clear framework for the systematic grouping of the five rRNA homology groups. Significant homogeneity exists within each rRNA group (Palleroni *et al.*, 1973), but DNA-DNA hybridization studies have revealed marked heterogeneity among the species within each rRNA group (Johnson & Palleroni, 1989). The results from the macrorestriction fragment analysis may reflect this low DNA homology and, if considered in the absence of phenotypic data, suggest that the isolates are only distantly related.

Similar diversity in macrorestriction profiles and poor correlation with phenotypic characters, especially carbon source assimilation ability, was observed within a collection of 17 phenotypically similar *P. stutzeri* isolates (Rainey *et al.*, 1994). Denny *et al.* (1988) also observed greater genotypic diversity among a collection of 17 *P. s. tomato* isolates (on the basis of RFLP analysis) than was evident by allozyme analysis, and Gillings & Fahy (1993) noted that restriction fragment diversity among *P. (Burkholderia) solanacearum* biovar N2 was greater than expected given the phenotypic uniformity of these isolates. Genomic analysis of *P. solanacearum* has also revealed a poor correlation between genotypic and phenotypic characteristics (Cook *et al.*, 1989, 1991; Hayward, 1991; Seal *et al.*, 1993; Gillings & Fahy, 1993). It is important that future studies begin to address the reasons for this poor correlation, because it is possible that the divergent patterns are the result of intragenomic recombination (possibly mediated by insertion sequences), and/or point mutations, and thus phylogenetic reconstructions based upon RFLP data may not be correct. This issue may be partly resolved by conducting a sequence analysis of conserved housekeeping genes and comparing the results with those obtained from RFLP data.

Bacteria capable of existing in diverse and variable environments typically exhibit greatest genetic diversity. Genetic diversity within a population of *P. (Burkholderia) cepacia* was shown to be positively correlated with habitat diversity (McArthur *et al.*, 1988); similarly, high diversity was detected within the *P. s. syringae* population – a plant pathogen with a broad host range (Denny *et al.*, 1988). Substantial genetic variation was also detected within a population of *Deinococcus radiopugans* isolated from soil (Masters *et al.*, 1991), *Rhizobium leguminosarum* biovar *phaseoli* isolated both from individual host plants in Mexico (Souza *et al.*, 1992) and from host plants isolated throughout the Americas (Piñero *et al.*, 1988), *Bacillus*

subtilis collected from the desert (Istock *et al.*, 1992), *Janthinobacterium lividum* from streams (Saeger & Hale, 1993), *Xanthomonas oryzae* from plant surfaces (Leach *et al.*, 1992) and *Erwinia chrysanthemi* isolated from a variety of different plant species taken from different geographical locations (Boccardo *et al.*, 1991). This contrasts markedly with the low genetic diversity often found within pathogen populations where, as a consequence of host or strain specificity, the pathogen occupies a narrow ecological niche. Analysis of the population structure of the highly specialized potato pathogen *Clavibacter michiganense* subsp. *sepidonicus* showed limited genetic diversity and an essentially clonal population structure (Mogen *et al.*, 1990), similarly, the *P. s. tomato* population was also shown to be genetically homogeneous (Denny *et al.*, 1988). Genetic diversity within populations of *Rhizobium leguminosarum* biovar *trifolii* is also reported to be limited (Harrison *et al.*, 1989).

Saprophytic epiphytes are unlikely to exhibit narrow host specificities, and given the genetic diversity of the sugar beet population and the diversity of leaf types and niches within leaves, it would be reasonable to expect the population of phyllosphere-inhabiting pseudomonads to be genetically diverse. Such an expectation is consistent with our experimental results. A genetically diverse population would have a greater potential for adaptation to environmental change (Ayala, 1982) and this is likely to be an essential requirement for survival on the leaf surface. While the sample size of the study population is too small to enable firm conclusions to be drawn about the fluorescent *Pseudomonas* population structure, the high intrapopulation genetic diversity combined with limited diversity per sampling occasion suggests a population which is comprised of a variety of apparently distantly related clones (genomic groups), each adapted to local conditions. Periods of intense selection could result in both the extinction of poorly adapted clonal lines and rapid proliferation of genotypes suited to the local conditions. The rate of turnover of clones (genomic groups) is unknown, but would appear to be less than 28 d, because in only one instance was an isolate from the same genomic group found on a later sampling occasion (genomic group IV contained isolates sampled on day 148 and day 183). The extent of the distribution of individual clones throughout the crop on each sampling occasion cannot be accurately determined given the sampling regime, nevertheless, on two instances isolates from the same genomic group were isolated from different replicate samples and from different leaf types, demonstrating that isolates of genomic groups II and III were present on at least two spatially isolated plants (within a field) and on more than a single leaf type. In fact further analysis of the 1990 field season data (Thompson *et al.*, 1993a) in light of the current study revealed that on day 114 the total *Pseudomonas* population on mature leaves (42% of the total bacterial population; 1.6×10^6 c.f.u. per unit) was comprised entirely of genomic group III (or IIIP) isolates. Similarly, of the total *Pseudomonas* population on immature leaves on sampling day 114, 69% (5.3×10^7 c.f.u. per unit) belonged to genomic group III (or IIIP).

The highly divergent macrorestriction fragment profiles of the different genomic groups suggest limited inter-genomic recombination within the population, but this may merely reflect the small sample size. The potential for recombination to occur in the sugar beet phyllosphere has been demonstrated both by *in situ* transfer of indigenous plasmids to selectable host isolates (Lilley *et al.*, 1994; M. J. Bailey unpublished data) and by the presence of a plasmid in isolate 114/60. Recombination may also have been responsible for the significant similarity between isolates 52/76 and 272/48, although the extraordinary similarity in protein profiles, combined with the fact that the chromosomes of the two isolates are of the same size, suggests the possibility that the diversity may have been generated by intragenomic recombination events.

This study has revealed the need to greatly improve our understanding of the population structure of fluorescent pseudomonads in the natural environment. Understanding the structure of a saprophytic *Pseudomonas* population is of prime importance for work concerned with the efficacy and safety of genetically modified bacteria intended for environmental release (Lenski, 1993), and will be invaluable in the continuing debate on the genetic structure of bacterial populations (Maynard Smith *et al.*, 1993). In addition, such studies will facilitate the development of integrated strategies for biotechnology where isolates can be selected on the basis of phenotype and genotype and with a knowledge of how these factors influence each other and interact with the environment.

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