Numerical Taxonomy of Phylloplane Bacteria Isolated from *Lolium perenne*

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(Received 27 July 1977; revised 15 September 1977)

Phenetic data on over 600 heterotrophic bacteria from green leaves of *Lolium perenne* s24 were collected and analysed using numerical taxonomic methods. Marker strains representing 60 taxa were included in the analyses. At similarity levels of 80 % or above, 74 % of the isolates were recovered in six major and 45 minor phena. Four of the major phena were equated with the taxa *Listeria grayi*[*murrayi*], *Pseudomonas fluorescens*, *Staphylococcus saprophyticus* and *Xanthomonas campestris*; the two unidentified phena contained pink, Gram-negative rods with polar flagella. Xanthomonads and pink chromogens formed high populations in May, xanthomonads and pseudomonads in July, xanthomonads in September and listeriae and staphylococci in October. It seems that despite the spatial proximity of leaves, litter and soil, each of these habitats contains independent bacterial communities with specific properties.

INTRODUCTION

The absence of satisfactory isolation methods and problems of classification and identification have hampered investigations of the bacterial flora of leaf surfaces. Workers have mainly used methods designed for the study of soil and litter micro-organisms and have classified bacteria into groups based upon differential characters of dubious value (Goodfellow, Austin & Dawson, 1976*a*). Identification using standard diagnostic schemes is also of limited value, for few strains can be identified beyond the genus level and others not at all (Dickinson, Austin & Goodfellow, 1975). It is now being appreciated that leaf surfaces have their own chemical and physical characteristics and may support unrecognized bacterial taxa. The lack of systematic studies on the composition of the phylloplane flora has hindered ecological work (Leben & Daft, 1965; Austin, Dickinson & Goodfellow, 1977).

Chromogenic bacteria, known to be numerically dominant in the phylloplane of many temperate plants, have often been classed together on the basis of a few morphological, biochemical, nutritional or pigmentation properties (Goodfellow *et al.*, 1976*a*). Such monothetic classifications are not satisfactory for the identification of isolates giving the same response to the 'key' characters but differing widely in other properties and they make comparisons between different investigations difficult. Thus, it is not always easy to distinguish between strains of *Cytophaga*, *Erwinia*, *Flavobacterium*, *Pseudomonas* and

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Xanthomonas and other yellow-pigmented bacteria frequently associated with plant matter. High populations of xanthomonads have been reported from *Lolium perenne* and *Trifolium repens* leaves (Stout, 1960; Dickinson *et al.*, 1975), flavobacteria from *Pinus* and grass leaves (Stout, 1958, 1960, 1961) and *Erwinia herbicola* from leaves of many Gramineae and Leguminosae (Last & Warren, 1972). Classifications based on pigmentation are particularly suspect for numerical phenetic surveys have shown that several different types of yellow chromogens exist (McMeekin, Stewart & Murray, 1972; Goodfellow, Austin & Dickinson, 1976b).

Numerical phenetic methods have successfully classified bacteria isolated from large mixed populations found in aquatic habitats (Reichelt & Baumann, 1973), in the rhizosphere (Graham, 1964) and in deciduous litter and mineral soil (Soumare, Losfield & Blondeau, 1973; Hissett & Gray, 1974) and have proved sensitive enough to distinguish taxa that would otherwise be put together because of current weaknesses in classification (Goodfellow, 1969; Goodfellow, Austin & Dickinson, 1976b). Numerical phenetic data also allow an objective choice of strains for comparative systematic and ecological investigations. Thus, representative soil isolates from clusters defined by Lowe & Gray (1972) were subsequently used in growth and competitive interaction work carried out in sterile soil (Lowe & Gray, 1973a, b).

In our previous studies bacteria were taken from healthy green leaves of *Lolium perenne* using a modified dilution plate procedure and over 600 randomly chosen isolates were identified using standard diagnostic schemes (Dickinson *et al.*, 1975). The predominant flora was provisionally identified to the genera *Chromobacterium*, *Corynebacterium*, *Pseudomonas* and *Xanthomonas*, but over 20% of the strains remained unidentified. In a pilot numerical taxonomic study, 113 of the 600 isolates were randomly selected, examined for 73 unit characters and recovered in five clusters defined at or above the 80% similarity level (S level) (Goodfellow *et al.*, 1976a). Given the success of the orientation study, all of the phylloplane isolates were examined for additional characters, and the data were analysed using numerical methods.

METHODS

Isolation and maintenance of the phylloplane isolates. *Lolium perenne* s24 (perennial rye grass) was collected from a moderately grazed pasture at Cockle Park Farm, Morpeth, Northumberland (Ordnance Survey grid reference NT217917) and dilution plates were prepared as described earlier (Dickinson *et al.*, 1975). The glucose yeast extract agar (GYEA) plates, prepared from samples collected on a seasonal basis, were incubated at 25 °C for 9 days. Six hundred and fifty bacterial colonies were randomly chosen from plates containing between 30 and 300 colonies and subcultured on to slopes of GYEA. Following further incubation for 7 days at 25 °C, stained smears were examined using Hucker's modification of the Gram stain (Hucker & Conn, 1923) and pure cultures were maintained on GYEA slopes at 5 °C and subcultured every 8 weeks. During the isolation and testing procedures 29 strains were lost. The history of the remaining 621 isolates is shown in Table 1.

Reference strains. The 67 reference cultures (Table 2) were maintained as above.

Collection of data. Each strain was examined for 135 unit characters (Tables 3 and 4). Media were inoculated from 5-day-old GYEA cultures and were incubated at 25 °C for 14 days unless otherwise stated. The tests were generally carried out once but were repeated when inconclusive results were obtained. Many of the tests used have been described elsewhere (Goodfellow *et al.*, 1976b); details of the remainder are given below.

Colony and micromorphology. Colony morphology was observed on GYEA plates after 5 days' incubation and pigmentation on GYEA supplemented with skimmed milk (5%, w/v) after 3 days. Fluorescein and pyocyanin pigments were detected on the media of King, Ward & Raney (1954). Bacterial size was determined from smears stained by Hucker's modification of the Gram stain (Hucker & Conn, 1923) and scored as small (< 2 µm long) or large (2 to 5 µm long). The presence of mycelium, densely staining intracellular areas and pleomorphism were also observed.

Biochemical and degradative tests. Oxidation of ethanol to acetic acid (Shimwell, Carr & Rhodes, 1960) was determined after 7 days' incubation, and the indole (method 2), methyl red and Voges-Proskauer tests (O'Meara's method) were examined after 3, 5 and 7 days respectively using the methods described

Table 1. *Test strains isolated from the phylloplane of Lolium perenne* S24

Date of isolation	No. of strains isolated		No. of isolates
	Leaf 2/5*	Leaf 3/6*	
16 May 1973	53 (A†)	47 (B†)	100
11 July 1973	125 (C)	125 (D)	250
5 September 1973	75 (E)	75 (F)	150
31 October 1973	70 (G)	51 (H)	121
	Total		621

* The second and third leaves were sampled, except on 31 October, when the fifth and sixth leaves were used.

† Code allocated to each sample series.

by Cowan & Steel (1965). Lactose fermentation was recorded after 5 days' incubation on MacConkey agar (Oxoid CM7); where colonies were naturally red or pink, MacConkey broth (Oxoid CM5) was used. Degradation of cellulose and urea were detected after 28 days' incubation by the methods of Stanier (1942) and Rustigan & Stuart (1941) respectively.

Tolerance tests. The ability of strains to grow on MacConkey agar and at pH 4.5 (Lemco broth, Oxoid CM15 amended with 1 M-HCl) was recorded after 7 days' incubation.

Coding of characters. The data were reduced to 135 mutually exclusive binary character states. Twelve characters with no differentiating value were eliminated and the final $n \times t$ matrix contained data for 688 organisms and 123 unit characters.

Computer analysis. The data were examined using the simple matching coefficient (S_{SM} ; Sokal & Michener, 1958), which includes both positive and negative matches, and sorted similarity matrices were obtained using unweighted average linkage clustering (UPGMA) (Sneath & Sokal, 1973). The homogeneity of the defined clusters was verified using the Jaccard coefficient (S_J ; Sneath, 1957) which counts positive matches only.

Identification of defined phena. Phena not containing reference strains were identified as far as possible using the diagnostic tables of Cowan & Steel (1965), keys in *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons, 1974) and the keys of Gordon, Haynes & Pang (1973).

RESULTS

Examination of data by the matching coefficient (S_{SM})

At the 80 to 85 % S level, 456 (74 %) of the phylloplane bacteria and 17 (25 %) of the reference strains were recovered in 51 phena (Fig. 1). The 17 reference strains helped in the identification of phena 3, 6, 8, 24, 28, 42 and 44; some of the remaining phena were identified using the standard schemes mentioned above. Six major phena (1, 3, 6, 7, 16 and 44), each containing at least 10 (1.6 %) of the isolates, were recognized from their various characteristics (Table 3).

Characteristics and identification of the major phena

Phenon 1 contains 11 pink chromogens which could not be identified using the diagnostic schemes. The strains were Gram-negative, polarly flagellate rods, about 3 μ m long, forming small (1 mm diam.), round, shiny, pink colonies on GYEA (Table 3). Glucose was attacked oxidatively, or not at all, and most strains decarboxylated arginine, lysine and ornithine, utilized malonate, degraded tributyrin but were oxidase-negative, non-proteolytic, did not reduce nitrate, and grew at 10 °C but not at 5 °C. L(–)-Arabinose, calcium lactate, capric acid, ethanol, D(–)-galactose, D(–)-glucose, glycerol, sodium acetate, sodium citrate, sodium fumarate, sodium gluconate, sodium glutamate, sodium malate, sodium malonate, sodium pyruvate and sodium succinate were utilized as sole carbon sources (Table 3). Pink chromogens with similar properties to those outlined above have been reported from leaf surfaces and identified as *Mycoplana rubra*, *Protaminobacter ruber* and *Vibrio extorquens* (Billing, 1976; de Vries & Derx, 1953).

Phenon 3 contains 122 isolates and *Xanthomonas campestris* NCPPB528, *X. begoniae*

Table 2. *Reference strains*

Taxon	Strain designation/source†	Isolated from
<i>Achromobacter anitratum</i>	NCIB9019	—
* <i>Aeromonas liquefaciens</i>	NCMB87	—
<i>Agrobacterium radiobacter</i> var. <i>radiobacter</i>	NCPPB2406	—
* <i>Agr. tumefaciens</i>	NCIB8150	—
<i>Arthrobacter globiformis</i> var. <i>aurescens</i>	NCIB8912	—
<i>Bordetella bronchiseptica</i>	NCTC452	Dog lung
* <i>Cellulomonas biazotea</i>	NCIB8077	—
<i>Chromobacterium typhi-flavum</i>	NCTC9381	Knee laceration (U.K.)
* <i>Chr. violaceum</i>	NCTC9757	Fresh water
<i>Citrobacter freundii</i>	NCTC6071	—
<i>Corynebacterium betae</i>	NCPPB375	<i>Beta vulgaris</i> L. (U.K.)
<i>Cor. fascians</i>	NCPPB1488	<i>Lathyrus odoratus</i> L. (U.K.)
<i>Cor. rathayi</i>	NCPPB797	<i>Dactylis glomerata</i> L.
<i>Enterobacter cloacae</i>	NCIB10101	—
<i>Erwinia amylovora</i>	NCPPB595	<i>Pyrus communis</i> L. (U.K.)
<i>E. ananas</i>	ICPB-EA133	—
<i>E. carnegieana</i>	ICPB-EC186	<i>Saguaro</i> spp. (U.S.A.)
<i>E. carotovora</i> var. <i>carotovora</i>	NCPPB312	<i>Solanum tuberosum</i> L.
<i>E. carotovora</i> var. <i>chrysanthemi</i>	NCPPB512	—
<i>E. cassavae</i>	ICPB-EC11	Uganda
<i>E. herbicola</i> var. <i>herbicola</i>	NCPPB656	<i>Malus sylvestris</i> (U.K.)
<i>E. herbicola</i> var. <i>herbicola</i>	NCPPB657	<i>Crataegus oxyacantha</i> L. (U.K.)
<i>E. nigrifluens</i>	NCPPB564	<i>Juglans regia</i> L. (U.S.A.)
<i>E. nimipressuralis</i>	ICPB-EN1	U.S.A.
<i>E. tracheiphila</i>	ICPB-ET5	<i>Cucumis</i> spp.
* <i>E. ureidovora</i>	NCPPB800	—
<i>Escherichia coli</i>	NCTC8620	Human diarrhoea (U.K.)
* <i>Flavobacterium aquatile</i>	NCIB8694	Deep well (U.K.)
* <i>F. meningosepticum</i>	NCTC10016	Human (U.S.A.)
* <i>F. pectinovorum</i>	NCIB9059	—
<i>F. suaveolens</i>	NCIB8992	Butter (Ireland)
<i>Hafnia alvei</i>	Emslie-Smith, PN	Human faeces (U.K.)
<i>Klebsiella aerogenes</i>	NCTC8172	—
<i>K. ozoneae</i>	NCTC5051	Amsterdam
<i>Obesumbacterium proteus</i>	NCIB8770	—
<i>Oerskovia turbata</i>	Lechevalier, 891	—
* <i>Pseudomonas acidovorans</i>	NCIB9681	—
* <i>P. aeruginosa</i>	NCIB8295	—
* <i>P. diminuta</i>	NCTC8545	Water (U.S.A.)
<i>P. fluorescens</i>		
Biotype A	Sneath, D123	—
Biotype B	Sneath, D1087	—
Biotype C	Sneath, D125	—
Biotype D	Sneath, D126	—
Biotype E	Sneath, D127	—
Biotype F	Sneath, D128	—
<i>P. maltophilia</i>	NCIB9204, NCTC10257	—
<i>P. marginalis</i>	NCPPB667	<i>Cichorium intybus</i> L.
<i>P. melophthora</i>	NCPPB42	<i>Malus sylvestris</i> Miller (U.K.)
<i>P. multivorans</i>	NCIB9085	—
<i>P. phaseolicola</i>	NCPPB52	<i>Phaseolus vulgaris</i> L. (Canada)
<i>P. putida</i>	CCEB520	—
<i>P. rubescens</i>	NCIB8768	—
* <i>P. stutzeri</i>	NCTC10457	—
<i>P. syringae</i>	NCPPB281	<i>Syringa vulgaris</i> L. (U.K.)
* <i>P. testosteroni</i>	NCPPB1969	—
<i>P. thomasii</i>	NCTC10893	—
<i>P. viridiflava</i>	NCPPB602	—
<i>Salmonella typhimurium</i>	NCTC5710	Copenhagen
<i>Serratia marcescens</i>	Laboratory culture	—
<i>Xanthomonas begoniae</i>	NCPPB2226	<i>Begonia</i> spp. (U.K.)
<i>X. campestris</i>	NCPPB528	<i>Brassica oleracea</i> L. (U.K.)
<i>X. hederae</i>	NCPPB2336	<i>Hedera canariensis</i> Willd (Netherlands)
<i>X. hyacinthi</i>	NCPPB599	<i>Hyacinthus orientalis</i> L.
<i>X. phaseoli</i>	NCPPB381	Canada
<i>X. rubrisubalbicans</i>	NCPPB1027	<i>Saccharum officinarum</i> L.
* <i>X. translucens</i>	NCPPB973	<i>Hordeum vulgare</i> L. (U.S.A.)

* Type strain.

† Sources. Culture collections outside the U.K.: CCEB, Culture Collection of Entomogenous Bacteria, Prague, Czechoslovakia; ICPB, International Collection of Phytopathogenic Bacteria, California, U.S.A. Others: Dr A. H. Emslie-Smith, Department of Microbiology, University of Newcastle upon Tyne; Dr M. P. Lechevalier, Institute of Microbiology, Rutgers University, U.S.A.; Professor P. H. A. Sneath, Department of Microbiology, University of Leicester.

Fig. 1. A simplified dendrogram showing the relationship between clusters based on the S_{SM} coefficient and unweighted average linkage clustering (UPGMA).

Table 3. *Characteristics of the major phena as percentages of positive reactions*

	Phenon number and name					
	1	3	6	7	16	44
	'Pink chromogens'	<i>Xanthomonas campestris</i>	<i>Staphylococcus saprophyticus</i>	'Pink chromogens'	<i>Listeria</i> spp.	<i>Pseudomonas fluorescens</i>
No. of strains ...	11	125	12	13	25	131
Colonial characters:						
Minute (< 1 mm)	100	0	0	100	0	0
Mucoid	0	96	0	0	0	0
Levan produced	0	74	0	0	0	0
Colonies, white	0	0	58	0	0	1
Colonies, cream	0	0	42	0	0	1
Colonies, pale yellow	0	10	0	0	60	98
Colonies, deep yellow	0	90	0	0	40	0
Colonies, pink	100	0	0	100	0	0
Fluorescent diffusible pigment	0	0	0	0	0	100
Stained preparations:						
Gram-positive	0	0	100	0	48	0
Cocci	0	0	100	0	0	0
Short rods (< 2 μ m)	0	100	0	0	100	100
Long rods (2 to 5 μ m)	100	0	0	100	0	0
Petrichous flagella	0	0	0	0	24	0
Polar flagella	100	64	0	69	0	100
Motility	100	64	0	69	24	100
Intracellular areas of high staining intensity	100	0	0	100	0	0
Biochemical tests:						
Arginine dihydrolase	100	12	0	100	0	99
Fermentative metabolism	0	0	100	0	84	0
Oxidative metabolism	73	70	0	61	0	100
Gluconate oxidation	0	0	0	0	0	98
Lactose fermentation	0	12	66	0	80	76
Lysine decarboxylase	73	3	0	69	0	1
Malonate utilization	100	0	0	61	0	100
Reduction, NO ₃ $\rightarrow$ NO ₂	0	18	66	23	8	0
Ornithine decarboxylase	82	3	0	61	0	5
Oxidase	0	12	0	0	0	95
Tolerance to:						
MacConkey agar	0	10	66	0	80	92
pH 4.5	0	0	58	0	0	0
NaCl (2 %, w/v)	0	58	100	8	100	100
NaCl (5 %, w/v)	0	0	100	0	28	95
NaCl (7.5 %, w/v)	0	0	50	0	0	8
Growth at:						
5 °C	18	11	0	8	100	100
10 °C	100	64	0	46	100	100
37 °C	0	0	100	0	0	2
Degradation of:						
Aesculin	0	32	0	0	100	2
Blood	0	5	66	0	8	95
Casein	9	27	0	0	8	79
Gelatin	0	69	66	0	24	100
Lecithinase	0	3	8	0	0	54
Starch	0	88	0	8	100	2
Tributylin	100	3	66	23	20	100
Tween 20	9	10	66	15	100	100
Tween 80	0	5	0	0	0	100
Tyrosine	0	5	0	0	4	97

Table 3 (cont.)

	Phenon number and name					
	1	3	6	7	16	44
No. of strains ...	'Pink chromogens'	<i>Xanthomonas campestris</i>	<i>Staphylococcus saprophyticus</i>	'Pink chromogens'	<i>Listeria</i> spp.	<i>Pseudomonas fluorescens</i>
	11	125	12	13	25	131
Sole carbon source (at 2 g l ⁻¹):						
<i>Cis</i> -aconitic acid	9	0	0	0	0	45
Adonitol	0	0	0	0	40	100
DL- α -Alanine	0	0	0	0	4	100
L(-)-Arabinose	100	1	0	15	100	100
D(-)-Arabinose	9	1	0	0	0	3
L(-)-Arginine.HCl	0	0	0	0	0	100
Calcium lactate	73	3	0	0	0	100
Capric acid	100	0	0	0	0	100
Caproic acid	0	0	0	0	0	95
Caprylic acid	0	0	0	0	0	2
Cellobiose	9	20	0	0	100	2
Chlorogenic acid	18	0	0	0	0	0
<i>meso</i> -Erythritol	0	3	0	0	40	100
Ethanol	100	3	0	8	0	6
D(-)-Fructose	0	10	0	8	100	100
D(-)-Galactose	100	6	0	0	28	100
D(-)-Glucose	91	96	66	0	100	100
Glycerol	100	64	0	0	100	100
L(-)-Histidine.HCl	0	2	0	0	0	100
<i>p</i> -Hydroxybenzoic acid	0	2	0	0	0	100
Inositol	0	2	0	8	0	100
Inulin	0	2	0	0	96	77
Lactose	0	0	0	0	96	0
L(-)-Leucine	0	3	0	8	0	100
Maltose	0	3	0	8	20	9
Mannitol	0	5	0	0	100	100
Mannose	0	5	0	0	100	100
Melezitose	0	3	0	0	40	6
DL-Norleucine	0	0	0	0	0	4
Pelargonic acid	0	0	0	0	0	100
L(-)-Proline	0	48	0	0	64	77
Putrescine	0	0	0	0	0	18
Raffinose	0	3	0	0	100	0
Ribose	0	6	0	8	48	100
Salicin	0	0	0	0	100	0
DL-Serine	0	0	0	0	0	100
Sodium acetate	100	2	0	8	0	92
Sodium benzoate	0	0	0	0	0	6
Sodium butyrate	9	0	0	0	0	100
Sodium citrate	100	3	0	0	96	100
Sodium formate	36	0	0	8	0	38
Sodium fumarate	100	2	0	23	48	100
Sodium gluconate	91	58	0	0	100	100
Sodium glutamate	100	2	0	46	92	92
Sodium malate	100	0	0	54	100	100
Sodium malonate	100	2	0	38	0	95
Sodium oxalate	0	0	0	0	0	1
Sodium pyruvate	100	2	0	8	0	100
Sodium succinate	100	3	0	46	100	98

Table 3 (cont.)

	Phenon number and name					
	1	3	6	7	16	44
	'Pink chromogens'	<i>Xanthomonas campestris</i>	<i>Staphylococcus saprophyticus</i>	'Pink chromogens'	<i>Listeria</i> spp.	<i>Pseudomonas fluorescens</i>
No. of strains ...	11	125	12	13	25	131
Sodium tartrate	0	0	0	15	0	1
Sorbitol	0	3	0	0	92	95
Spermine .4HCl	0	3	0	0	0	92
Sucrose	0	3	17	0	100	100
Trehalose	0	16	0	0	100	100
DL-Tryptophan	0	0	0	0	0	56
L(-)-Tyrosine	0	2	0	0	0	100
DL-Valine	0	2	0	0	0	100
D(+)-Xylose	0	3	0	0	100	92

Negative characters common to the phenetic groups. *Colony characteristics*: Central colonial bodies, Dry-granular, Rhizoidal, Spreading, Green, Orange, Red, Violet, Pyocyanin. *Stained preparations*: Mycelium, Pleomorphism. *Biochemical tests*: Indole production, Methyl red, Phenylalanine deaminase, Voges-Proskauer. *Degradation of*: Cellulose, Sodium hippurate. *Growth at 42 °C*. *Utilization as sole carbon source*: Pimelic acid, Starch.

NCPB2226 and *X. hederæ* NCPB2336. Since the last two are subjective synonyms of *X. campestris* (Dye & Lelliott, 1974) this name was preferred for phenon 3 isolates. Strains were Gram-negative rods, < 2 µm long, producing yellow, mucoid colonies on GYEA. Motility, when recorded, was by means of a single polar flagellum. Glucose was attacked oxidatively, or not at all, and most isolates were oxidase-negative, did not reduce nitrate, and did not decarboxylate arginine, lysine or ornithine. Most strains produced levan, degraded starch and gelatin, grew at 10 °C, but utilized only glucose, glycerol and sodium gluconate of the sole carbon sources tested (Table 3).

Phenon 6 contains 11 isolates and *Pseudomonas melophthora* NCPB42. However, our culture of the latter was a fermentative Gram-positive coccus showing few of the properties expected for pseudomonads (Stanier, Palleroni & Doudoroff, 1966). The isolates were identified as *Staphylococcus saprophyticus* by their colonial and micromorphology, ability to ferment glucose and inability to degrade starch or aesculin or decarboxylate ornithine (Cowan & Steel, 1965; Baird-Parker, 1974*a, b*). The strains grew at 37 °C in the presence of 5 % (w/v) sodium chloride; most grew on glucose as sole carbon source but, apart from sucrose, no other sole carbon source supported growth (Table 3).

Phenon 7 contains 13 pink chromogens which could not be identified using the diagnostic schemes. In many respects, the strains resembled those in phenon 1 except that they generally did not readily degrade any of the organic compounds tested or grow on the various sole carbon sources tested (Table 3).

Phenon 16 contains 25 isolates tentatively identified as *Listeria* (Seeliger & Welshimer, 1974) by their morphology and ability to degrade aesculin, and by their inability to produce indole or hydrolyse casein, gelatin and urea. The organisms were Gram-variable rods, < 2 µm long, motile by means of a few peritrichous flagella, forming yellow colonies on GYEA. Glucose and lactose were attacked fermentatively, starch and Tween 20 were degraded but nitrate was not reduced. Growth occurred in 2 % (w/v) sodium chloride, at 5 and 10 °C and on a wide range of sole carbon sources including L(-)-arabinose, cello-

biose, D(-)-fructose, D(-)-glucose, glycerol, inulin, lactose, mannitol, mannose, raffinose, salicin, sodium citrate, sodium gluconate, sodium glutamate, sodium malate, sodium succinate, sorbitol, sucrose, trehalose and D(+)-xylose.

Phenon 44 includes 125 isolates and the six reference strains of *Pseudomonas fluorescens*. The isolates were Gram-negative, oxidative rods, motile by means of a single polar flagellum, forming colonies which exhibited the fluorescent, diffusible pigment fluorescein. In addition, the strains were oxidase-positive, decarboxylated arginine, degraded casein, gelatin, tributyrin, Tweens 20 and 80, and tyrosine and utilized malonate and a wide range of compounds as the sole source of carbon and energy (Table 3). They also grew at 5 and 10 °C and in the presence of 5 % (w/v) sodium chloride. This cluster was identified as *P. fluorescens* on the basis of the properties listed above and the inclusion of the marker strains.

Minor phena

Twenty-six per cent of the phylloplane isolates were recovered in the 45 minor phena. The characteristics of these are listed in tables which can be obtained from the senior author. The unidentified phena were mainly labelled on the basis of pigmentation (Fig. 1); the identified taxa are considered below.

Only two Gram-positive spore-bearing rods were found, forming phenon 5. However, they could not be identified further with the keys of Gordon *et al.* (1973).

Yellow Gram-negative, oxidase-negative, fermentative rods, generally exhibiting gliding motility (Perry, 1973) but not degrading cellulose or chitin were identified as *Flexibacter* species (Leadbetter, 1974). These isolates were recovered in phenon 8 and in phena 17 to 25. However, most flexibacteria were recovered in two aggregate clusters defined at or above the 70 % S level (Fig. 1). *Flavobacterium aquatile* NCIB8694 and *Flavobacterium pectinovorum* NCIB9059 were recovered in phena 8 and 24 respectively. However, the status of these species is not clear for the reference strains exhibit gliding motility (Weeks, 1974; Goodfellow *et al.*, 1976b) and should not be classified in the genus *Flavobacterium*. Most of the flexibacteria grew at 5 and 10 °C and on a wide range of sole carbon sources (Table 5).

Phena 26 to 37 contain Gram-positive or Gram-variable, fermentative, non-sporing, irregular or pleomorphic rods, provisionally labelled 'coryneform' bacteria. Most of the 'coryneform' taxa fell into one of three aggregate clusters defined at or above the 75 % S level (Fig. 1). *Corynebacterium betae* NCPPB375 was recovered in phenon 28. Chemo-taxonomic (Keddie & Cure, 1977) and numerical phenetic data (Jones, 1975) indicate that *C. betae* should be reclassified in the coryneform taxon *Curtobacterium* (Yamada & Komagata, 1972). The 'coryneform' bacteria grew at 10 °C, most degraded aesculin, tributyrin and Tweens 20 and 80 and utilized a wide range of sole carbon sources.

Phena 39 and 45 contain short, non-motile, oxidative, Gram-negative rods which are oxidase-negative and were identified as *Acinetobacter* spp. (Lautrop, 1974; Reyn, 1974; Pagel & Seyfried, 1976).

Phenon 40 contains four strains loosely associated with *Pseudomonas maltophilia* NCIB9204, NCTC10257 and *Pseudomonas multivorans* NCIB9085. The isolates were Gram-negative, non-fluorescent, polarly flagellated, oxidative rods, able to degrade aesculin, casein, gelatin, lecithin, tributyrin, Tweens 20 and 80 and utilize a wide range of carbon compounds as the sole source of carbon and energy. The phenon appears to be intermediate between *P. maltophilia* and *P. multivorans* (Stanier *et al.*, 1966) and may represent an undescribed species.

Phenon 42 contains five isolates, *Chromobacterium typhi-flavum* NCTC9381 and *Erwinia herbicola* NCPPB656. Strains were Gram-negative, fermentative rods, motile by means of 3 to 6 peritrichous flagella, able to reduce nitrate and utilize a wide range of carbon compounds as the sole source of carbon and energy. The strains in phenon 43, which shared

Table 4. *Distinguishing characters for the six major phena*

	Phenon number and name					
	1	3	6	7	16	44
	'Pink chromogens'	<i>Xanthomonas campestris</i>	<i>Staphylococcus saprophyticus</i>	'Pink chromogens'	<i>Listeria</i> spp.	<i>Pseudomonas fluorescens</i>
No. of strains ...	11	125	12	13	25	131
Colonial characters:						
Minute (< 1 mm)	+	—	—	+	—	—
Mucoid	—	+	—	—	—	—
Pigment	Pink	Deep yellow	White/cream	Pink	Yellow	Pale yellow
Fluorescent diffusible pigment	—	—	—	—	—	+
Stained preparations:						
Gram stain	—	—	+	—	d	—
Micromorphology	Long rod	Short rod	Coccus	Long rod	Short rod	Short rod
Biochemical tests:						
Oxidation (O)/Fermentation (F)	o/—	o/—	F	o/—	F	o
Arginine dihydrolase	+	d	—	+	—	+
Gluconate oxidation	—	—	—	—	—	+
Malonate utilization	+	—	—	d	—	+
Oxidase	—	d	—	—	—	+
Degradation of:						
Aesculin	—	d	—	—	+	—
Starch	—	d	—	—	+	—
Tributyrin	+	—	d	d	d	+
Tween 20	—	—	d	d	+	+
Tween 80	—	—	—	—	—	+
Tyrosine	—	—	—	—	—	+
Tolerance to:						
NaCl (2 %, w/v)	—	d	+	—	+	+
NaCl (5 %, w/v)	—	—	+	—	d	+
Growth at:						
37 °C	—	—	+	—	—	—
Sole carbon source (at 2 g l ⁻¹):						
Adonitol	—	—	—	—	d	+
DL- α -Alanine	—	—	—	—	—	+
L(—)-Arabinose	+	—	—	d	+	+
L(—)-Arginine.HCl	—	—	—	—	—	+
Capric acid	+	—	—	—	—	+
Caproic acid	—	—	—	—	—	+
Cellobiose	—	d	—	—	+	—
meso-Erythritol	—	—	—	—	d	+
Ethanol	+	—	—	—	—	—
D(—)-Fructose	—	—	—	—	+	+
D(—)-Galactose	+	—	—	—	d	+
D(—)-Glucose	+	+	d	—	+	+
Glycerol	+	d	—	—	+	+
L(—)-Histidine.HCl	—	—	—	—	—	+
p-Hydroxybenzoic acid	—	—	—	—	—	+
Inositol	—	—	—	—	—	+
Inulin	—	—	—	—	+	d
Lactose	—	—	—	—	+	—
L(—)-Leucine	—	—	—	—	—	+

Table 4 (cont.)

		Phenon number and name					
		1	3	6	7	16	44
		'Pink chromogens'	<i>Xanthomonas campestris</i>	<i>Staphylococcus saprophyticus</i>	'Pink chromogens'	<i>Listeria</i> spp.	<i>Pseudomonas fluorescens</i>
No. of strains	...	11	125	12	13	25	131
Mannitol		—	—	—	—	+	+
Mannose		—	—	—	—	+	+
Pelargonic acid		—	—	—	—	—	+
Raffinose		—	—	—	—	+	—
Ribose		—	—	—	—	d	+
Salicin		—	—	—	—	+	—
DL-Serine		—	—	—	—	—	+
Sodium acetate		+	—	—	—	—	+
Sodium butyrate		—	—	—	—	—	+
Sodium citrate		+	—	—	—	+	+
Sodium fumarate		+	—	—	d	d	+
Sodium gluconate		+	d	—	—	+	+
Sodium glutamate		+	—	—	d	+	+
Sodium malate		+	—	—	d	+	+
Sodium malonate		+	—	—	d	—	+
Sodium pyruvate		+	—	—	—	—	+
Sodium succinate		+	—	—	d	+	+
Sorbitol		—	—	—	—	+	+
Spermine.4HCl		—	—	—	—	—	+
Sucrose		—	—	d	—	+	+
Trehalose		—	d	—	—	+	+
L(—)-Tyrosine		—	—	—	—	—	+
DL-Valine		—	—	—	—	—	+
D(+)-Xylose		—	—	—	—	+	+

+, More than 90 % of strains positive; —, more than 90 % of strains negative; d, strains 11 to 89 % positive.

a similarity of over 80 % with *E. herbicola* (Fig. 1), were identified as *Klebsiella* spp. These isolates were Gram-negative, non-motile, fermentative rods, Voges-Proskauer-positive and, like *E. herbicola*, used a wide range of carbon compounds for energy and growth. The two strains forming phenon 47 were identified as *Micrococcus luteus* (Baird-Parker, 1974c) and those in phenon 50 as *Staphylococcus* spp. (Baird-Parker, 1974a).

Miscellaneous isolates

Twenty-six per cent (165) of the isolates were not recovered in any of the phena defined at or above the 80 % S level. The unclassified strains included 22 Gram-positive cocci, 2 actinomycetes and 12 pseudomonads (Stanier *et al.*, 1966), one of which fitted the description of *Pseudomonas cepacia* (Palleroni & Doudoroff, 1972). The remaining isolates were Gram-negative rods, 21 of which were related to the 'pink chromogens' of phena 1 and 7.

Characters associated with the phena

Characters potentially useful for the discrimination of the phena are shown in Tables 4 and 5.

Table 5. *Diagnostic table for the identification of the minor phena*

Phenon	Colour*	Gram reaction	Cell shape†	Oxidation (o)/ Fermentation (F)	Oxidase	Arginine dihydrolase	NaCl (2 %, w/v)	Lactose fermentation	Aesculin	Casein	Gelatin	Lecithin	Starch	Tyrosine	L(-)-arabinose	D(-)-galactose	Inositol	Lactose	Maltose	Mannitol	D(+)-xylose
2	p	-	r	O	-	+	-	-	-	-	-	-	D	-	-	-	+	D	D	-	-
4	c	-	r	O	+	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	+
5	w	+	r	O	D	-	D	D	-	-	-	-	-	-	-	-	-	+	-	-	-
8	y	-	r	F	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-
9	c/py	-	r	F	-	-	D	-	+	-	-	-	+	-	-	-	-	-	-	D	-
10	py	-	r	O	-	-	-	-	-	-	-	D	+	-	-	-	-	-	-	-	D
11	y	-	r	F	-	-	+	-	+	-	-	-	+	-	-	-	-	-	+	-	-
12	y	-	r	F	-	-	+	-	+	D	-	-	+	-	+	-	-	+	-	+	+
13	w/c	-	r	O	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+
14	y	-	r	O	-	-	D	D	D	+	+	-	D	-	D	-	+	-	+	+	+
15	o	-	r	O	-	-	D	-	+	-	-	-	-	-	-	-	+	-	-	+	-
17	py	+	r	F	-	-	+	+	+	-	+	-	+	-	+	-	-	D	+	+	+
18	py	+	r	F	-	-	+	+	D	+	+	-	D	-	+	+	-	+	+	+	+
19	y	+/-	r	F	-	-	+	D	+	-	+	D	+	-	+	+	D	+	+	+	+
20	py	-	r	F	-	-	+	D	-	-	D	-	D	-	+	+	D	+	+	+	+
21	y	+	r	O	-	-	+	+	D	D	-	-	-	-	+	+	-	+	+	+	+
22	y	-	r	F	-	-	-	-	+	-	D	-	-	+	+	+	-	D	+	-	+
23	y	-	r	F	-	-	-	D	+	-	D	-	D	D	+	+	-	+	+	-	+
24	y	-	r	F	-	-	-	-	+	-	-	-	+	-	+	D	-	D	+	-	+
25	y	-	r	F	+	-	D	-	+	-	D	-	+	-	+	+	-	+	+	+	+
26	y	-	r	O	-	-	+	+	D	+	-	-	D	-	+	+	+	+	+	+	+
27	y	-	r	O	-	-	+	D	+	-	-	-	D	-	D	+	+	+	+	+	+
28	y	+/-	r	F	-	-	+	+	D	+	-	-	D	-	+	+	+	+	+	+	+
29	y/o	-	r	F	-	-	+	D	+	+	+	+	+	-	+	+	D	D	+	+	+
30	y	-	r	F	-	-	+	D	+	+	-	+	+	-	+	+	-	+	-	-	-
31	c	+	r	O	-	-	+	+	+	D	+	-	+	-	+	+	D	D	+	+	+
32	w	+	r	O	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+
33	o	-	r	F	-	-	-	-	+	-	-	-	+	-	+	-	-	D	-	+	-
34	o	-	r	F	-	-	+	-	+	-	-	-	D	-	+	+	-	+	+	-	+
35	o	-	r	F	-	-	-	-	D	-	-	-	+	-	+	D	-	+	+	-	+
36	o	-	r	F	-	-	-	+	D	-	-	-	+	-	+	D	-	-	+	-	+
37	o	-	r	O	-	-	-	-	-	-	-	-	-	-	+	+	-	+	D	-	+
38	y	-	r	O	+	+	-	+	D	-	-	-	-	-	+	+	-	+	+	-	-
39	p	-	r	O	-	-	D	+	D	-	-	-	D	-	+	-	D	-	+	+	-
40	py	-	r	O	-	-	+	-	+	+	+	D	+	+	-	+	-	+	D	-	+
41	y	-	r	F	-	-	+	-	+	-	-	-	+	-	+	-	+	D	D	+	+
42	y	-	r	F	-	-	+	+	D	D	-	-	-	-	+	+	+	-	+	+	+
43	w	-	r	F	-	-	+	+	+	-	-	-	-	-	+	+	-	+	+	+	+
45	p	-	r	O	-	+	+	-	D	-	-	-	+	-	+	+	-	+	+	+	+
46	y	-	r	F	-	-	+	-	-	D	+	+	+	-	+	+	+	+	-	+	+
47	y	+	c	O	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+
48	py	-	r	O	-	D	+	-	-	-	-	-	-	-	+	+	-	-	+	+	+
49	y	+/-	r	F	-	-	+	D	+	-	D	-	+	D	+	+	D	+	+	+	+
50	y	+	c	F	-	-	+	D	+	D	D	D	+	D	D	D	D	D	+	+	D
51	py	-	r	O	-	-	+	-	-	+	+	+	-	+	-	D	D	-	+	-	-

+, More than 80 % of strains positive; -, less than 20 % of strains positive; D, 21 to 79 % of strains positive.

* c, cream; o, orange; p, pink; py, pale yellow; w, white; y, yellow.

† r, rods; c, cocci.

Table 6. Temporal distribution of the major phena

Results indicate the numbers of isolates, out of the total of 621, of each taxon recorded on each date. Numbers in parentheses indicate the frequency of each taxon as a percentage of the total isolate on each date.

	Distribution in 1973				% of total strains examined
	16 May	11 July	5 September	31 October	
'Pink chromogens' (phenon 1)	100 (11)	0	0	0	1.7
<i>Xanthomonas campestris</i> (phenon 2)	32 (28)	16 (14)	46 (40)	6 (5)	19.4
<i>Staphylococcus saprophyticus</i> (phenon 6)	0	0	22 (2)	78 (7)	1.8
'Pink chromogens' (phenon 7)	80 (8)	20 (2)	0	0	2.1
<i>Listeria</i> spp. (phenon 16)	0	0	20 (4)	80 (16)	4.0
<i>Pseudomonas fluorescens</i> (phenon 44)	0	100 (55)	0	1 (1)	20.0
Total no. phena	10	13	16	20	

General characteristics of the isolates

All of the test strains were catalase-positive, did not produce hydrogen sulphide, oxidize alcohol, degrade cellulose, chitin, sodium hippurate or urea, or utilize adipic acid, geraniol, pimelic acid, urea or valeric acid as sole carbon sources.

Most of the strains were Gram-negative rods (90 %), pigmented (81 %), mainly yellow (58 %), grew at 5 °C (61 %) and 10 °C (75 %) but not at 37 °C (12 %) and 42 °C (1 %). Approximately half of the strains were motile, mainly pseudomonads with polar flagella, and about half degraded aesculin (41 %), casein (50 %), gelatin (39 %), tributyrin (64 %), Tween 20 (58 %) and Tween 80 (60 %). Fewer degraded starch (28 %) or reduced nitrate (19 %). Mainly negative results were recorded for the Voges-Proskauer (2 %), methyl red (2 %), indole (2 %) and phenylalanine deaminase (2 %) tests.

The majority of strains produced acid from glucose, 45 % aerobically and 25 % anaerobically. Nearly all of the sugars and carbon sources tested were attacked by at least some of the cultures: L(-)-arabinose (60 %), fructose (70 %), galactose (55 %), glycerol (58 %), mannitol (53 %), mannose (68 %), sodium gluconate (60 %), sodium malate (58 %), sucrose (59 %), trehalose (61 %) and xylose (61 %) were most frequently utilized; D(-)-arabinose (8 %), caprylic acid (4 %), chlorogenic acid (8 %), DL-norleucine (4 %), pimelic acid (4 %), sodium benzoate (6 %), sodium succinate (2 %), sodium tartrate (3 %) and starch (3 %) were only sparingly used.

Temporal distribution of the isolates

The diversity of the bacteria isolated from the phylloplane of *Lolium perenne* increased progressively throughout the sampling period (Table 6). *Xanthomonas campestris* occurred consistently on both newly expanded and ageing leaves throughout the season. The other relatively common taxa were only isolated in any quantity on one or two occasions. This suggests that either the composition of the populations was rapidly shifting, which may be correlated with large increases in total numbers (Dickinson *et al.*, 1975), or alternatively the sampling system may have been inadequate to cover spatial variations in the leaf populations. Support for the former conclusion comes from the similarity between the September and October populations which were from leaves of two different positions on the plants.

DISCUSSION

The present findings reinforce the view that numerical taxonomic methods can effectively record variation in large unknown mixed populations of bacteria. If the bacterial flora of natural habitats contains strains with independent and correlated characters then taxonomic methods can be used to detect and define representative samples of the constituent taxa. In this first numerical phenetic survey of leaf surface bacteria, 74 % of the *Lolium* isolates were recovered in six major and 45 minor phena. There was sufficient consistency within, and differences between, phena for characters to be extracted from the data and weighted for identification.

It is now realized that test errors can distort similarity coefficients (Sneath & Johnson, 1972) and that even 'standard' tests are not always reliable (Sneath & Collins, 1974). However, in ecological studies some taxonomic principles may sometimes be overlooked so that additional isolates can be studied. In this survey, test error and reproducibility studies were not done; omissions which should be made good in future investigations. Nevertheless, the recovery of well-defined and homogeneous phena suggests that test error was within acceptable limits. Only 17 of the marker strains (25 %) were recovered in defined phena and even then only four phena were identified. This result, like those from previous studies (Goodfellow *et al.*, 1976*a*), merely illustrates the difficulties in selecting strains for the identification of taxa in analyses of large mixed populations of bacteria. However, as more becomes known of the species composition of the leaf surface flora, the easier the choice of marker strains will become. Clearly, future studies on leaf surface bacteria should include good representatives of the genera *Acinetobacter*, *Corynebacterium*, *Curtobacterium*, *Flexibacter*, *Listeria*, *Micrococcus*, *Pseudomonas*, *Staphylococcus*, *Xanthomonas* and of less well established taxa containing yellow chromogens (Goodfellow *et al.*, 1976*b*). Phena which do not contain marker strains are often identified using conventional keys (Pfister & Burkholder, 1965; Lowe & Gray, 1972); however, this expedient can be of limited value, as in the present study, for distinct phena can be keyed out to the same taxon. These difficulties emphasize that natural habitats may contain bacteria that previously either went unrecognized or were 'dumped' into heterogeneous groups after a cursory examination of a few 'important' characters.

Nearly half of the *Lolium* isolates were identified as *Pseudomonas fluorescens*, *Staphylococcus saprophyticus*, *Xanthomonas campestris*, *Listeria* spp. or as pink chromogens (clusters 1 and 7). Although it is difficult to compare numerical phenetic classifications with those based on only a few subjectively chosen characters, it should be noted that Stout (1960) recorded large numbers of *Pseudomonas* spp., *Xanthomonas* spp. and pink chromogens (flavobacteria) from *Lolium* leaves. *Pseudomonas fluorescens* strains have been isolated from the leaves of *Phaseolus vulgaris* L. (Teliz-Ortiz & Burkholder, 1960), *Fagus* (Jensen, 1971), *Prunus* spp. (Barclay & Crosse, 1974) and *Pinus* (Goodfellow *et al.*, 1976*a*) and seem to be indigenous to leaf surfaces. *Listeriae* are widely distributed in nature (Gregorio & Eveland, 1975) and both *Listeria monocytogenes* and *Listeria murrayi* have been isolated from decaying vegetation (Welshimer & Meredith, 1971; Welshimer & Donker-Voet, 1971; Weiss & Seeliger, 1975). Unlike *L. monocytogenes* strains those from *Lolium* were not haemolytic, did not degrade Tween 80 or produce acetoin and can be provisionally allotted to the *L. grayi*/*murrayi* group (Seeliger & Welshimer, 1974). There are no previous reports of *Staph. saprophyticus* or *X. campestris* from the phylloplane but it is likely that such strains have been misclassified in the past. Pink, Gram-negative, motile rods with a polar flagellum have been found in association with plant material (Billing, 1976) and, like representatives of some of the minor clusters, need further study.

It seems that at any one time only a few bacterial species predominate on the leaf surface. Thus, xanthomonads and pink chromogens formed large populations in May but were replaced by *Listeriae* and *Staphylococci* by October. Jensen (1971) noted that fluorescent

pseudomonads were common on *Fagus* leaves during early summer but declined rapidly in August, when large numbers of non-pigmented rods were found. The reasons for such fluctuations are not known but they may reflect changes in the nutrient regime, moisture, temperature and other ecological parameters. These findings parallel the situation that has been determined for the fungal community in these habitats (Dickinson & Wallace 1976) and they suggest that the phylloplane is a very selective habitat for microbial growth. The recovery of large populations of *Xanthomonas campestris* throughout the growing season suggests that they form part of the indigenous flora of *Lolium* leaves. The ability of these strains to produce extracellular levan *in vitro* may be significant since phytopathogenic pseudomonads often produce polysaccharide [gums that block the vessels of susceptible hosts (Agrios, 1969). In general, phylloplane isolates are proficient in utilizing sugars, sugar alcohols and inorganic salts as sole sources of carbon for energy and growth; fructose, galactose, glucose and sucrose have been detected in leaf exudates (Tukey, 1971) and may be important to phylloplane bacteria *in vivo*.

Despite their spatial proximity, leaves, litter and soil apparently contain independent bacterial communities with specific properties (Hissett & Gray, 1974). It is evident from this and earlier work (Goodfellow *et al.*, 1976*a, b*) that phylloplane bacteria are predominantly Gram-negative, chromogenic rods, many of which can be identified as *Pseudomonas* and *Xanthomonas*. Pigmentation may confer resistance to ultraviolet radiation in sunlight (Mathews & Sistrom, 1960) or desiccation (Grinstead & Lacey, 1973). By contrast the soil flora generally includes large populations of Gram-positive, non-chromogenic bacteria classified as *Arthrobacter*, *Bacillus* and *Streptomyces* (Stout, 1960; Goodfellow, 1969; Jensen, 1971; Holm & Jensen, 1972; Lowe & Gray, 1972) though Gram-negative bacteria are common in some grassland soils (Stout, 1961) and in the rhizosphere (Rovira & Brisbane, 1967). Bacteria on litter may occupy an intermediate position. Hissett & Gray (1974) recovered large populations of *Arthrobacter* and *Pseudomonas* from *Fraxinus* and *Quercus* litter.

Little is known of the significance of this bacterial flora to the plant or to other indigenous leaf surface micro-organisms. However, the numerical classification of the bacterial flora of *Lolium perenne* leaves has made it possible to select strains objectively for interaction studies. Thus, representative strains have been shown to be antagonistic to *Drechslera dictyoides* (Drechsler) Shoemaker, the agent of net blotch disease of *L. perenne* (Austin *et al.*, 1977). Similarly, the highlighting of characters for the identification of phylloplane bacteria will make it possible to monitor bacterial populations on green and senescent leaves and their responses to seasonal factors, to pathogen damage and to man-made factors, such as fertilizers and pesticides.

The authors thank Dr J. R. Leece for his invaluable help with the computation and Dr D. Jones for helpful consultation. B.A. thanks the Natural Environment Research Council for a Research Studentship.

Additional data regarding the characteristics of isolates in the minor phenotypes may be obtained from B.A.

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