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Short communication

Identification of two serological flagellar types (H1 and H2) in *Pseudomonas syringae* pathovars

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Abstract

Flagellar antigen specificity was studied for the species *Pseudomonas syringae*, *P. viridiflava* and *P. cichorii*. After checking their motility, bacteria were reacted against six polyclonal antisera containing anti-O (LPS) and anti-H (flagellar) antibodies by indirect immunofluorescent staining. Two distinct flagellar serotypes (H1 and H2) were described. The distribution of H1 and H2 serotypes was then determined for a collection of 88 phytopathogenic *Pseudomonas* strains. Serotype H1 was possessed by *P. syringae* pv. *aptata* (12 strains), *P. s.* pv. *helianthi* (2), *P. s.* pv. *pisi* (11), and *P. s.* pv. *syringae* (13). Serotype H2 was possessed by *P. cichorii* (2), *P. s.* pv. *delphinii* (1), *P. s.* pv. *glycinea* (4), *P. s.* pv. *lacrymans* (1), *P. s.* pv. *mori* (1), *P. s.* pv. *morsprunorum* (10), *P. s.* pv. *persicae* (1), *P. s.* pv. *phaseolicola* (8), *P. s.* pv. *tabaci* (10) and *P. s.* pv. *tomato* (1). *P. viridiflava* (5) revealed HI, H2 and untyped flagella. The following isolates were untypable by the H1/H2 system: *P. corrugata* (3), *P. fluorescens* (2), *P. tolaasii* (1). H1/H2 serotypes distribution is not linked to *P. syringae* O-serogroups. On the other hand, H1/H2 distribution seems remarkably linked to the new genospecies of the *P. syringae* group.

Abbreviations: CFBP = French Collection of Phytopathogenic Bacteria, Angers, France; ICMP = International Collection of Micro-organisms from Plants, Auckland, New-Zealand; NCPPB = National Collection of Plant Pathogenic Bacteria, Harpenden, Great Britain.

The *Pseudomonas syringae* group, identified by the criteria of Palleroni [1984] (fluorescent pigment, no oxidase nor arginine-dihydrolase, hypersensitive reaction on tobacco) consists of 49 pathovars [Young *et al.*, 1991]. Earlier studies of the serological properties of *P. syringae* showed that the O-antigens (cell wall lipopolysaccharides) were the dominant antigens [(Guillorit and Samson, 1993 a and b]. O-groupings were found to have limited value in the identification of individual pathogens in the case of pathovars belonging to the same O-serogroup. For instance, serological detection of *P. syringae* pv. *pisi* occasionally gives false positive results because some *P. syringae* pv. *syringae* strains cross-react with APTPIS antisera [Grondeau *et al.*, 1992]. For this reason, the differentiation of pathogens using other kinds of antigens was investigated. In particular, the use of bacterial flagella (H) antigens of genera such as *Salmonella* is well developed [Le Minor, 1984]. Because of its pathogenicity to human beings, the type-species of the *Pseudomonas* genus (*P. aeruginosa*), has also been well studied and H serotypes were described [Lanyi, 1970]. In the present study, serological types of flagella within *P. syringae* pathovars were established and compared to some phytopathogenic *Pseudomonas* species. Immunofluorescent staining was chosen because fixation of the antibodies on the cell wall or on the flagella could be directly observed.

A total of 88 *Pseudomonas* isolates were studied (Table 1). Strains were identified by methods described by Lelliott and Stead [1987] and Hildebrand *et al.* [1988]; specifically levan, oxidase, pectolysis, argi-

Table 1. Isolates of *Pseudomonas* spp.

CFBP strain	Alternate designation	Host plant	Origin	Year	Flagellar serotype
P. cichorii					
1373	28–1	*Lactuca scariola*	France	1972	H2
2101^T	NCPPB 943^T	*Cichorium endivia*	– *	1929	H2
P. corrugata					
–	Lopez 53	*Lycopersicum esculentum*	Spain	–	unknown
–	Lopez 113	*L. esculentum*	Spain	–	unknown
–	Lopez 903FS	*L. esculentum*	Spain	–	unknown
P. fluorescens					
2022	584–1	*Allium sativum*	France	1976	unknown
2569	M32–1	*Prunus armeniaca*	France	1979	unknown
P. syringae pv. *aptata*					
1617^T	NCPPB 871^T	*Beta vulgaris*	USA	1959	H1
1625	NCPPB 873	*B. vulgaris*	USA	1959	H1
1906	1357–1	*B. vulgaris*	France	1979	H1
2042	SA33–5	*B. vulgaris*	France	1981	H1
2134	SA84–6	*B. vulgaris*	France	1981	H1
2135	SB30–3	*B. vulgaris*	France	1982	H1
2279	JA100–1	*B. vulgaris*	France	1981	H1
2280	JB47	*B. vulgaris*	France	1982	H1
2471	PD 565	*B. vulgaris*	Netherlands	1985	H1
2472	PD 196	*B. vulgaris*	Netherlands	1979	H1
2473	PD 197	*B. vulgaris*	Netherlands	1979	H1
2507	PD 202	*B. vulgaris*	Netherlands	1979	H1
P. syringae pv. *delphinii*					
2215^T	ICMP 52B^T	*Delphinium sp.*	New Zealand	1957	H2
P. syringae pv. *glycinea*					
1518	NCPPB 2070	*Glycine max*	USA	1962	H2
1563	NCPPB 2753	*G. max*	France	1974	H2
2214^T	ICMP 2189^T	*G. max*	New Zealand	1968	H2
3361	Fett A29–2	*G. max*	USA	1975	H2
P. syringae pv. *helianthi*					
1732	NCPPB 2639	*Helianthus annuus*	Canada	1974	H1
2149	SA51–1	*H. annuus*	France	1981	H1
P. syringae pv. *lacrymans*					
1644	NCPPB 1096	*Cucumis sativus*	Hungary	1957	H2
P. syringae pv. *mori*					
2453	1900B	*Morus sp.*	France	1984	H2
P. syringae pv. *morsprunorum*					
1565	NCPPB 2756	*Prunus domestica*	France	1965	H2
1566	768–7	*P. cerasus*	France	1968	H2
1647	NCPPB 1781	*P. avium*	Italy	–	H2
1650	NCPPB 2422	*P. domestica*	Switzerland	1965	H2
1658	NCPPB 2320	*P. domestica*	Rumania	1969	H2
1741	NCPPB 2787	*P. avium*	Greece	1961	H2
2115	G88–9	*P. cerasus*	France	1975	H2
2119	E13–20	*P. cerasus*	France	1974	H2
2332	EM78	*P. cerasus*	Great Britain	1976	H2
2351^T	NCPPB 2995^T	*P. domestica*	USA	–	H2

Table 1. Continued

CFBP strain	Alternate designation	Host plant	Origin	Year	Flagellar serotype
P. syringae pv. *persicae*					
1316	NCPPB 2324	*P. persica*	France	1970	H2
P. syringae pv. *phaseolicola*					
1390^T	NCPPB 52^T	*Phaseolus vulgaris*	Canada	1949	H2
1429	IPO 38	*P. vulgaris*	Netherlands	–	H2
1508	Hala	*P. vulgaris*	Germany	1972	H2
1652	NCPPB 1103	*P. vulgaris*	Hungary	1956	H2
1659	NCPPB 2435	*Vigna radiata*	USA	1971	H2
1662	NCPPB 604	*Phaseolus coccineus*	Great Britain	1958	H2
1667	NCPPB 1647	*Dolichos* sp.	Tanzania	1964	H2
1743	NCPPB 1098	*Phaseolus vulgaris*	New Zealand	1953	H2
P. syringae pv. *pisi*					
2105^T	ICMP 2452^T, R2	*Pisum sativum*	New Zealand	1969	H1
2702	NCPPB 2222, R1	*P. sativum*	Italy	1969	H1
2704	Taylor 870A, R3	*P. sativum*	USA	1975	H1
2706	Taylor 895A, R4	*P. sativum*	USA	1975	H1
2707	Taylor 974B, R5	*P. sativum*	USA	1978	H1
2710	Taylor 1683, R6	*P. sativum*	Hungary	1956	H1
3283	Taylor 277, R1	*P. sativum*	Australia	1938	H1
3288	Taylor 2491B, R1	*P. sativum*	Great Britain	1989	H1
3522	SG30–5	*P. sativum*	France	1987	H1
–	SH207–14	*P. sativum*	France	1988	H1
–	Si319A7	*P. sativum*	France	1989	H1
P. syringae pv. *syringae*					
602	NCPPB 2775	*Malus sylvestris*	France	1964	H1
1579	NCPPB 2769	*Prunus cerasus*	France	1974	H1
1669	NCPPB 294	*Populus* sp.	Great Britain	–	H1
1679	NCPPB 1041	*Piper nigrum*	Brasil	1958	H1
1685	NCPPB 2264	*Zea mays*	Yugoslavia	1965	H1
1894	896–2	*Z. mays*	France	1978	H1
2009	ICMP 849	*Prunus avium*	New Zealand	1974	H1
3077	20–27–37	*Pyrus communis*	France	1983	H1
3388	Psi3	*Vicia sativa*	France	1992	H1
–	SF15–10	*Pisum sativum*	France	1986	H1
–	SH243A1K	*P. sativum*	France	1988	H1
–	Si30A1K	*P. sativum*	France	1989	H1
–	SJ77–7	*P. sativum*	France	1990	H1
P. syringae pv. *tabaci*					
1503	BBL Dpt 5	*Nicotiana tabacum*	–	–	H2
1615	NCPPB 79	*N. tabacum*	USA	1935	H2
1621	NCPPB 1866	*N. tabacum*	–	–	H2
1699	NCPPB 1408	*N. tabacum*	Hungary	1959	H2
1721	NCPPB 1238	*N. tabacum*	Malaysia	1962	H2
1774	NCPPB 1234	*N. tabacum*	Zambia	1962	H2
1775	NCPPB 2729	*Glycine max*	Australia	1972	H2
1783	NCPPB 1919	*Nicotiana tabacum*	–	1966	H2
1786	NCPPB 2728	*Glycine max*	Australia	1972	H2
2106^T	NCPPB 1427	*Nicotiana tabacum*	Hungary	1959	H2

Table 1. Continued

CFBP strain	Alternate designation	Host plant	Origin	Year	Flagellar serotype
P. syringae pv. *tomato*					
2212^T	NCPPB 1106^T	*Lycopersicum esculentum*	Great Britain	1960	H2
P. tolaasii					
2068^T	NCPPB 2192^T	*Agaricus bisporus*	Great Britain	1965	unknown
P. viridiflava					
1141	NCPPB 1249	*Chrysanthemum morifolium*	Great Britain	1962	H2
1466	T6–1	*Lycopersicum esculentum*	France	1972	unknown
1468	V9b	*Prunus domestica*	France	1972	H1
2107^T	NCPPB 635	*Phaseolus* sp.	Switzerland	1927	H2
2555	M71–1	*Pyrus communis*	France	1979	H2

^{T}: type strain; *: no data; R: race

nine and tobacco tests (LOPAT), hydrolysis of casein, tyrosine, and aesculin; and acidification or alkalinisation of the carbohydrates in a mineral medium. Results of tests on these strains confirmed their authenticity.

Antisera were produced in rabbits inoculated with bacterial suspensions (10^8 cells per ml) washed in phosphate buffer (0.01 M, pH 7.2) and treated with formalin (0.5%) after 24 h growth in shaken YP broth (yeast extract 0.3% w/v, peptone 0.5% w/v). Four intravenous injections were administered to the rabbits within two weeks. When the antiserum titre was high enough (1/16 by Ouchterlony double diffusion and 1/1600 by immunofluorescent staining), the animals were bled. Injections were sometimes repeated after one month's rest. This way, OH antisera were produced that contained anti-O and anti-H antibodies.

The reacting antigens were prepared as follows. Strains were grown on YP semi-solid agar (0.3% agar w/v). Motility was ascertained using a phase-contrast microscope, and only motile strains were tested by immunofluorescent staining [Faure *et al.*, 1977]. The indirect reaction was performed on suspensions of 10^7cells per ml (20 μl), deposited on a microscope slide. The rabbit sera under test were used for the first step of the reaction, and a fluorescent serum (antirabbit IgG H and L chains antibodies, labelled with fluorescein isothiocyanate) for the second step. All rinsings and dilutions were performed with phosphate buffer (0.01 M, pH 7.2). Reactions were observed in epifluorescence under UV light, which revealed bacterial cells and their flagella (Fig. 1).

Among the various antisera prepared for previous studies [Samson and Saunier, 1987; Guillorit and Samson, 1993 a and b], some antisera gave non-specific

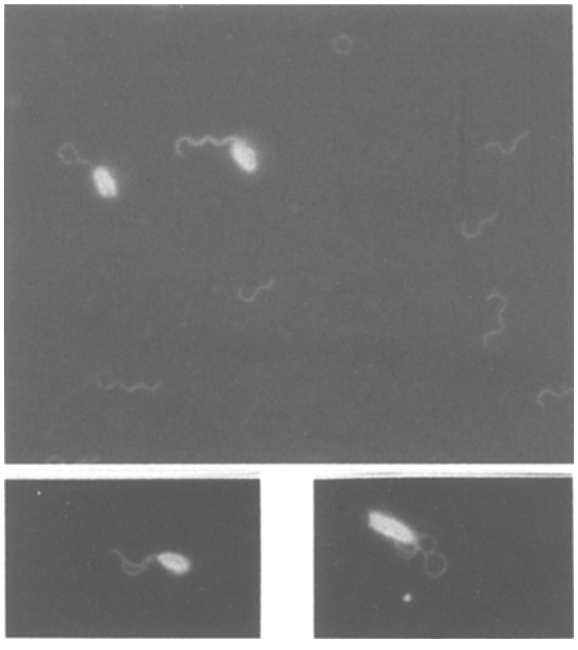

Fig. 1. Pseudomonas syringae pv. *pisi* (2105^T) cells stained with immunofluorescent technique (indirect method, antiserum S184–1). Notice the cell bodies, polar flagella and free flagella. The three pictures show various observations of the polar flagella after glass slide adsorption. Microscope photography at 1,000 magnification.

bands observed by Ouchterlony double-diffusion, suggesting the existence of flagellar antigens. Six antisera revealed anti-H antibodies : S32 against 1373 (*P. cichorii*), S223–2 against 1732 (*P. s.* pv. *helianthi*), S183–2 against 2115 (*P. s.* pv. *morsprunorum*), S184–1 against 2105^T (*P. s.* pv. *pisi*), S115–2 against 1894 (*P. s.* pv. *syringae*) and S191–2 against 2212^T (*P. s.* pv.

Table 2. Definition of the two flagellar serotypes (H1 and H2) by immunofluorescent staining method with six antisera raised against *Pseudomonas cichorii* and *P. syringae* pathovars

Pseudomonas strain	Antiserum					
	S32	S223–2	S183–2	S184–1	S115–2	S191–2
	Directed against					
	1373	1732	2115	2105^T	1894	2212^T
P. cichorii 1373	+*	–	+	–	–	+
P.s. pv. *helianthi* 1732	–	+	–	+	+	–
P.s. pv. *morsprunorum* 2115	+	–	+	–	–	+
P. s. pv. *pisi* 2105^T	–	+	–	+	+	–
P.s. pv. *syringae* 1894	–	+	–	+	+	–
P.s. pv. *tomato* 2212^T	+	–	+	–	–	+
Flagellar serotype	H2	H1	H2	H1	H1	H2

^{T}: type-strain; * +: fluorescent flagella, –: absence of fluorescent flagella

tomato). All six antisera were cross-tested with the six bacteria (Table 2). The strains 1732, 2105^T and 1894 showed fluorescent flagella indistinctly by the use of their three respective antisera. This behaviour defined the H1 serotype. The strains 1373, 2115 and 2112^T reacted with the three remaining antisera that defined a second H2 serotype. Only two mutually exclusive serotypes were observed.

The distribution of the two serotypes H1 and H2 was tested among 88 strains of *Pseudomonas* spp. Each strain was tested by immunofluorescent staining using the six-antiserum panel. 39 strains, belonging to four *P. syringae* pathovars and *P. viridiflava*, presented H1 serotype. 42 strains, belonging to nine *P. syringae* pathovars, *P. cichorii* and *P. viridiflava* presented an H2 serotype (Table 1). Although motile, seven strains showed no H1 nor H2 flagella reactions. Antisera should be produced against them to establish whether they would define additional serotypes. Several isolates of *P. syringae* were not included in the H-typing because they remained non-motile despite numerous attempts at enhancement trials on semi-solid agar.

H serotypes significantly cluster strains belonging to distinct *P. syringae* pathovars: H1 for pvs *aptata*, *helianthi*, *pisi* and *syringae* ; H2 for the other pvs. Moreover, they are not randomly distributed in the pathovars. H clustering must be compared to the different genospecies of the *P. syringae* group recently described by Gardan *et al.* [1994] and Shaffik [1994] by quantitative DNA:DNA homology: H1 for the genospecies *P. syringae* (including pv. *aptata*, pv. *pisi* and pv. *syringae*) and *P. helianthi*; H2 for *P. savastanoi* (including pv. *glycinea*, pv. *lacrymans*, pv. *morsprunorum*, pv. *phaseolicola* and pv. *tabaci*) and *P. tomato* (including pv. *persicae* and pv. *tomato*). The two H serotypes appear therefore linked to distinct taxonomical entities. They contribute to express genomic parenty between Pseudomonads. Thus, it has not been possible to find specific H antigens that could distinguish *P. s.* pv. *pisi* from *P. s.* pv. *syringae*.

H serotypes overlap the O-serogroups described for *P. syringae* pathovars [Samson and Saunier, 1987; Guillorit and Samson, 1993b]. For instance, pv. *glycinea* (O-serogroup APTPIS), pv. *lacrymans* (O-serogroup LAC), pv. *morsprunorum* (O-serogroups MOP1 and MOP2), pv. *phaseolicola* (O-serogroup PHA) and pv. *tabaci* (O-serogroup TAB) share the same H2 flagellar type. Earlier reports all indicated that heat-labile antigens were common between some *P. syringae* pathovars [Lovrekovich and Klement, 1961; Lucas and Grogan, 1969; Coleno *et al.*, 1970; Otta and English, 1971; Pastushenko and Simonovich, 1979]. It seems probable the common antigens reported were associated with flagella.

In contrast to the agglutination, double-diffusion and ELISA reactions, the fluorescent antibody technique allows the differentiation of bacterial soma and flagella at the morphological and serological levels simultaneously, and thereby, the specific analysis of flagellar H-antigens, even with OH-antisera. However, because flagellar preparations can be readily contaminated with small amounts of LPSs [Ada *et al.*, 1964], it is difficult to raise specific anti-H sera. Instead of anti-H sera, the use of anti-whole cell sera with immunofluorescent staining to visualize flagella is recommended.

The same anti-whole cell sera are convenient for O-serogrouping *P. syringae* strains by double-diffusion, if antigenic preparations have been heated to eliminate cross-reactions of H antisera.

References

Ada GL, Nossal GLV, Pye J and Abbot A (1964) Antigens in Immunity. I. Preparation and properties of flagellar antigens from *Salmonella adelaide*. Australian Journal of Experimental Biology and Medical Science 42: 267–270

Coleno A, Hingand L and Barzic MR (1970) Contribution à l'étude de *Pseudomonas phaseolicola* (Burk) Dowson. Annales de Phytopathologie 2: 199–207

Faure M, Dupouey P and Morelec MJ (1977) La technique de l'immunofluorescence et les réactions immunoenzymatiques, 566 p, Maloine, Paris.

Gardan L, Shafik H and Bollet C (1994) Taxonomy of some pathovars of *Pseudomonas syringae*. Plant Pathogenic Bacteria, Versailles (France) Ed. INRA, Paris 1994 (Les colloques, 66): 419–420

Grondeau C, Saunier M, Poutier F and Samson R (1992) Evaluation of physiological and serological profiles of *Pseudomonas syringae* pv. *pisi* for pea blight identification. Plant Pathology 41: 495–505

Guillorit C and Samson R (1993a) Serological specificity of the lipopolysaccharides, the major antigens of *Pseudomonas syringae*. Journal of Phytopathology 137: 157–171

Guillorit C and Samson R (1993 b) Serological study of four pathovars of *Pseudomonas syringae*: *Ps. syr. aptata*, *Ps. syr. tabaci*, *Ps. syr. mors-prunorum* and *Ps. syr. phaseolicola*. Journal of applied Bacteriology 74: 683–687

Hildebrand DC, Schroth MN and Sands DC (1988) *Pseudomonas*. In: Schaad NW (ed) A Laboratory Guide for Identification of Plant Pathogenic Bacteria (pp. 60–80) American phytopathological Society, St. Paul Minnesota

Lanyi B (1970) Serological properties of *Pseudomonas aeruginosa*. II. Type-specific thermolabile (flagellar) antigens. Acta microbiologica Academiae Scientarium Hungaricae 3: 303–310

Lelliott RA and Stead DE (1987) Methods for the Diagnosis of Bacterial Diseases of Plants. Blackwell Scientific Publications, Oxford

Le Minor L (1984) Salmonella. In: Krieg NR and Holt JG (eds) Bergey's Manual of Systematic Bacteriology (pp. 427–458) Williams and Wilkins, Baltimore

Lovrekovich L and Klement Z (1961) Species-specific antigens of *Pseudomonas tabaci*. Acta microbiologica Academiae Scientarium hungaricae 3: 303–310

Lucas LT and Grogan RG (1969) Serological variation and identification of *Pseudomonas lachrymans* and other phytopathogenic *Pseudomonas* nomenspecies. Phytopathology 59: 1908–1912

Otta JD and English H (1971) Serology and pathology of *Pseudomonas syringae*. Phytopathology 61: 443–452

Pastushenko L T and Simonovich I D (1979) (Groupes sérologiques des bactéries phytopathogènes du genre *Pseudomonas*. 1. Parenté antigénique intraspécifique). Mikrobiologicheski Zhurnal 41: 222–229. Translated by T. Babilliot, INRA Versailles France

Samson R and Saunier M (1987) Désignation de références sérologiques pour six sérogroupes de pathovars de *Pseudomonas syringae* sur la base de leur lipopolyoside. Bull. OEPP/EPPO Bull. 17: 165–171

Saunier M, Malandrin L and Samson R (submitted) Description of 23 O-serogroups within *Pseudomonas syringae* (*sensu lato*) and *P. viridiflava*

Shafik HL (1994) Taxonomie des *Pseudomonas* phytopathogènes du groupe de *Pseudomonas syringae*: étude phénotypique et génotypique. Thèse Univ. Angers

Young JM, Bradbury JF, Davis RE, Dickey RS, Ercolani GL, Hayward AC and Vidaver AK (1991) Nomenclatural revisions of plant pathogenic bacteria and list of names 1980–1988. Review of Plant Pathology 70: 211–221