Genetic Analysis of Plant Pathogenic Bacteria in the Genus *Clavibacter* Using Allozyme Electrophoresis

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(Received 26 April 1988; revised 5 August 1988)

Allozyme electrophoresis was used to examine the relationships among species of *Clavibacter*, a genus of bacteria mostly causing gummosis in various graminaceous hosts. Allelic profiles were determined at 20 enzyme loci for 28 strains representing *C. iranicum*, *C. rathayi*, *C. tritici*, three subspecies of *C. michiganense*, *Clavibacter* sp. from *Lolium rigidum*, and ‘*Corynebacterium agropyri*’. The genetic differences between species were at least 70% and between strains within a species up to 35% except for the subspecies of *C. michiganense*, which differed by up to 75%. The implications of these results for the classification of these *Clavibacter* spp. is discussed.

INTRODUCTION

The genus *Clavibacter* Davis, Gillaspie, Vidaver and Harris 1984 contains plant pathogenic coryneform bacteria characterized by the presence of 2,4-diaminobutyric acid as a cell wall component (Davis *et al.*, 1984; Riley 1987). Within this genus there is a group of species which produce distinctive infections of grasses: *Clavibacter iranicum* (Carlson and Vidaver 1982) Davis, Gillaspie, Vidaver and Harris 1984 and *Clavibacter tritici* (Carlson and Vidaver 1982) Davis, Gillaspie, Vidaver and Harris 1984 on *Triticum* spp. L.; *Clavibacter rathayi* (Smith 1913) Davis, Gillaspie, Vidaver and Harris 1984 on *Dactylis glomerata* L.; *Clavibacter* sp. on *Lolium rigidum* Gaud. The *Clavibacter* sp. from *L. rigidum* has occasionally been found in other grass species in infected *L. rigidum* pasture (Chatel *et al.*, 1979; Riley, 1987) but for the purposes of this paper will be referred to as *Clavibacter* sp. ex *Lolium*. The typical symptom of infection by these pathogens is gummosis of the grass inflorescence before it emerges from the boot. *C. iranicum*, *C. tritici* and *Clavibacter* sp. ex *Lolium* require for infection of their hosts nematode vectors in the genus *Anguina* (Sabet, 1954; Price *et al.*, 1979); the same is likely to be true of the other pathogens, given the similarity of the symptoms and their specific adhesion to *Anguina* spp. (I. T. Riley & A. C. McKay, unpublished). ‘*Corynebacterium agropyri*’, a bacterium which caused gummosis of *Agropyron* spp. Gaertn., has recently been reisolated from 30- and 40-year old herbarium specimens (Murray, 1986) and although pathogenicity could not be confirmed, the bacterium does adhere specifically to *Anguina* spp. (I. T. Riley & A. C. McKay, unpublished) and does contain 2,4-diaminobutyric acid in its cell walls (T. D. Murray, personal communication), and therefore fits with this group.

The taxonomic relationships within this group of plant pathogens now classified as *Clavibacter* (Davis *et al.*, 1984) have been examined in a number of studies, and proposed classifications have ranged from classifying each of the pathogens as pathovars of a single species, *Clavibacter michiganense* (Smith 1910) Davis, Gillaspie, Vidaver and Harris 1984

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through to maintaining each as a distinct species (Bradbury, 1973; Carlson & Vidaver, 1982; Davies *et al.*, 1984; Dye & Kemp, 1977; Lazar, 1968; Masuo & Nakagawa, 1970; Riley, 1987; Sabet, 1954; Seiler, 1983). The nomenclature of Davis *et al.* (1984), the most recent treatment, has been used in this paper. No single comparative study has included strains of all the pathogens likely to belong to the genus *Clavibacter*.

The only comparative study including the *Clavibacter* sp. from *L. rigidum* and hosts associated with infected *L. rigidum* pasture (Riley, 1987) found that, based on serological data, it was quite distinct from *C. rathayi*, the name which has been commonly used for this pathogen on the basis of an inadequate identification without comparison with recognized strains (Bird & Stynes, 1977). No comparative study that includes '*Corynebacterium agropyri*' has been reported. Clarification of the relationship of *Clavibacter* sp. ex *Lolium* and '*Corynebacterium agropyri*' to the other *Clavibacter* spp. is still required.

The aim of the work reported here was to examine the genetic relationships among strains in the genus *Clavibacter*, with particular emphasis on the pathogens causing gummosis of grasses, using allozyme electrophoresis. This technique has been most useful in the resolution of taxonomic problems at the species level in eukaryotic organisms (Richardson *et al.*, 1986). Although the technique has been used in relatively few bacterial studies, Ochman *et al.* (1983) listed some advantages of multilocus electrophoresis over other methods for measuring genetic variation in bacteria.

METHODS

The *Clavibacter* strains used are presented in Table 1; fuller details of these strains were presented by Riley (1987). Each strain was grown on agar slopes (Riley, 1987) for 6 d. The bacterial growth was suspended in 9 ml sterile water and diluted to an optical density of 0.1 at 600 nm. Liquid medium [containing, g l⁻¹: glucose, 10; Casamino acids (Difco), 2; yeast extract (Difco), 2; K₂HPO₄, 2; MgSO₄·7H₂O, 0.3], having been autoclaved (121 °C, 15 min) before the addition of the glucose, was dispensed into replicate flasks (50 ml in 250 ml Erlenmeyer flasks) for each strain and inoculated with 1 ml bacterial suspension. The cultures were grown for 48 h at 25 °C on an orbital shaker at 200 r.p.m. Cells were harvested by centrifugation to give at least 200 µl pellet and were washed three times in sterile 0.02 M-phosphate-buffered saline at 5 °C. Excess liquid was removed after centrifugation for 5 min at 12000 g. An equal volume of cold lysing solution (Richardson *et al.*, 1986) was added to the pellet, and the mixture sonicated in the cold for 15 s in three bursts of 5 s each. The homogenates were centrifuged at 4 °C at 8000 g for 5 min. The supernatants were dispensed into capillary tubes and stored at -20 °C until used for electrophoretic analysis.

Table 1. *Clavibacter* strains examined using allozyme electrophoresis

Species	Laboratory number and source*
<i>Clavibacter iranicum</i>	CS13, PDDCC 3496 ^T
<i>Clavibacter michiganense</i>	
subsp. <i>insidiosum</i>	CS20, NCPPB 1109
subsp. <i>michiganense</i>	CS25, NCPPB 2979 ^T
subsp. <i>nebraskense</i>	CS24, NCPPB 2581 ^T
<i>Clavibacter rathayi</i>	CS5, PDDCC 2571; CS6, PDDCC 2572; CS7, PDDCC 2573; CS15, NCPPB 80; CS18, NCPPB 797; CS26, NCPPB 2980 ^T
<i>Clavibacter</i> sp.	
ex <i>Lolium</i> (S.A.)	CS1, A.F. Bird, Manoora; CS2, A.F. Bird, Murray Bridge; CS14, Murray Bridge, 1983; CS33, Port Gawler, 1984; CS34, Wharminda, 1984
ex <i>Lolium</i> (W.A.)	CS3, A.F. Bird, Kantanning; CS28, WSM 188; CS29, WSM 454; CS30, WSM 447; CS31, WSM 455; CS32, WSM 456
<i>Clavibacter tritici</i>	CS4, PDDCC 2624; CS12, PDDCC 2628; CS16, NCPPB 471; CS17, NCPPB 255; CS21, NCPPB 1857 ^T ; CS22, NCPPB 1953
' <i>Corynebacterium agropyri</i> '	CS35, T.D. Murray, CA-1

* PDDCC, Plant Diseases Division Culture Collection, Plant Diseases Division, DSIR, Mt Albert Rd, Private Bag, Auckland, New Zealand; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; WSM, Western Australian Department of Agriculture, Baron-Hay Ct, South Perth, WA 6151, Australia.

Allozyme electrophoresis was done on Cellogel (Chemetron, Milan) according to the procedures given in Richardson *et al.* (1986). Electrophoresis on any day used samples thawed on that day. A total of 35 enzymes were stained for, of which 20 gave staining of sufficient intensity and resolution for reliable genetic interpretation. The enzymes scored, with an abbreviation and the Enzyme Commission designation, were adenylate kinase, AK (EC 2.7.4.3); alkaline phosphatase, AP (EC 3.1.3.1); acetylsterase, EST (EC 3.1.1.6); glucose-6-phosphate dehydrogenase, G6PD (EC 1.1.1.49); glucose-phosphate isomerase, GPI (EC 5.3.1.9); aspartate aminotransferase, GOT (EC 2.6.1.1); glutathione reductase, GSR (EC 1.6.4.2); hexokinase, HK (EC 2.7.1.1); isocitrate dehydrogenase, IDH (EC 1.1.1.42); leucine aminopeptidase, LAP (EC 3.4.11.1); mannose-6-phosphate isomerase, MPI (EC 5.3.1.8); purine nucleoside phosphorylase, NP (EC 2.4.2.1); peptidase (phenylalanine proline), PEPD (EC 3.4.13.9); phosphoglycerate kinase, PGK (EC 2.7.2.3); phosphoglycerate mutase, PGAM (EC 5.4.2.1); 6-phosphogluconate dehydrogenase, 6PGD (EC 1.1.1.44); pyruvate kinase, PK (EC 2.7.1.40); triosephosphate isomerase, TPI (EC 5.3.1.1); UDP-glucose pyrophosphorylase, UGPP (EC 2.7.7.9); uridine monophosphate kinase, UMPK (EC 2.7.4.4). For each pair of strains, the percentage of allelomorphs out of 20 that were different was calculated to yield a difference matrix. This matrix was used to construct a phenogram by average linkage cluster analysis (Sneath & Sokal, 1973).

RESULTS

The 28 strains under investigation fell into 14 electrotypes. Table 2 shows the allelic profiles for the 14 electrotypes of *Clavibacter* strains; the strains within each electrototype had identical profiles for all 20 loci. The strains are grouped into electrotypes A1 to A3 for *Clavibacter* sp. ex *Lolium*, B1 to B3 for *Clavibacter michiganense*, C for *Clavibacter iranicum*, D for '*Corynebacterium agropyri*', E1 to E3 for *Clavibacter rathayi* and F1 to F3 for *Clavibacter tritici*.

The phenogram derived by average linkage cluster analysis from the matrix of percentage differences among the 14 groups of strains is presented in Fig. 1. The differences between electrotypes belonging to different species ranged from 70% to 95% and between electrotypes within a species from 5% to 35% except for the subspecies of *Clavibacter michiganense*, which differed by 45% to 75%.

Fig. 1. Phenogram showing relationships among *Clavibacter* electrotypes based on average linkage cluster analysis of the percentage differences between electrotypes (strains which constitute each electrototype are given in Table 2).

Table 2. Allelic profiles for groups of *Clavibacter* strains

Species	Clavibacter* electrotype	Strains	Alleles† at enzyme locus:																			
			AK	AP	EST	GOT	G6PD	GPI	GSR	HK	IDH	LAP	MPI	NP	PEPD	PGAM	6PGD	PGK	PK	TPI	UGPP	UMPK
Clavibacter sp. ex <i>Lolium</i>	A1	CS1 CS2 CS14 CS34	b	c	a	a	a	b	b	b	a	b	a	e	a	a	a	c	d	c	f	a
	A2	CS33	a	c	a	a	a	b	b	a	b	a	f	a	a	a	c	d	c	c	f	a
	A3	CS3 CS28 CS32	b	d	a	a	a	b	b	b	a	b	a	e	a	a	c	d	c	c	f	a
Clavibacter <i>michiganense</i>	B1	CS24	b	b	e	c	a	g	a	d	f	c	b	a	d	e	e	e	e	b	c	c
	B2	CS25	e	a	e	c	a	g	a	c	f	c	b	b	c	f	d	c	f	b	c	c
	B3	CS20	d	f	a	b	a	f	a	b	f	d	b	a	a	e	e	e	e	a	b	c
Clavibacter <i>iranicum</i>	C	CS13	e	c	c	d	d	d	a	a	b	b	d	d	e	b	b	a	c	f	e	b
	D	CS35	d	e	d	b	d	c	b	d	b	c	d	e	c	c	b	b	d	g	d	d
<i>Corynebacterium</i> <i>agropyri</i>	E1	CS5 CS15	d	e	d	e	b	c	b	c	e	b	b	d	e	g	c	b	a	d	a	b
	E2	CS18	d	e	b	e	b	c	b	c	e	a	b	d	e	e	c	a	b	d	a	b
	E3	CS6 CS7 CS26	d	e	b	d	b	c	b	c	c	a	b	e	e	e	c	b	b	d	a	b
Clavibacter <i>tritici</i>	F1	CS4 CS17	c	g	d	d	c	e	c	c	e	d	c	b	b	d	b	d	d	e	c	c
	F2	CS12 CS22	c	g	d	f	c	e	c	c	d	d	c	c	e	d	b	d	d	e	c	c
	F3	CS16 CS21	c	g	d	f	c	d	c	c	d	d	c	c	e	d	b	d	d	e	c	c

* '*Corynebacterium agropyri*' contains 2,4-diaminobutyric acid in its cell walls, as do other plant pathogenic coryneforms in the genus *Clavibacter*.

† Alleles are designated alphabetically in order of mobility, with 'a' being the most cathodal.

Within *Clavibacter* sp. ex *Lolium* there were three electrotypes, A1, A2 and A3. The electrotypes A1 and A2 consisted solely of strains from Western Australia whereas the South Australian strains fell in two electrotypes, A1 and A2. The Western Australian strains (A3) differed by about 18% from the South Australian strains (A1 and A2) due to differentiation at three enzyme loci, AK, GPI and NP. The electrotypes A1 and A2 (consisting of a single strain, CS33) differed by 5% from the other South Australian strains (A3) due to a single enzyme locus, AP.

The *Clavibacter rathayi* strains consisted of three electrotypes, E1, E2 and E3, with a maximum difference of 35%. The *C. rathayi* strains CS5 and CS15, from the same original isolation though procured from different culture collections (Table 1), gave identical profiles.

Similarly the *Clavibacter tritici* strains included three electrotypes, F1, F2 and F3, with a maximum difference of 25% between pairs. Again two strains, CS4 and CS17, which had originated from the same isolation (Table 1) were in the one electrotypes.

Consistent with haploid organisms, no heterozygous phenotypes were observed; however, the enzyme bands produced by the *C. michiganense* strains were diffuse and more difficult to score.

DISCUSSION

The classification of *Clavibacter iranicum*, *C. tritici*, *C. rathayi*, *Clavibacter* sp. ex *Lolium* and '*Corynebacterium agropyri*' as distinct species is consistent with the data collected in this study. The differences within each of these species were no more than 35% but at least 70% between these species.

The difficulty arises with the *Clavibacter michiganense* subspecies, which had differences of up to 75% between *C. michiganense* subsp. *insidiosum* and *C. michiganense* subsp. *michiganense*. The *C. michiganense* subspecies were originally named as separate species but were subsequently considered to be sufficiently similar to be classified as members of one species (Dye & Kemp, 1977; Carlson & Vidaver, 1982).

For many higher organisms a large body of data shows that the level of genetic divergence between populations within a species is not normally greater than 15% and in birds as little as 5% (Richardson *et al.*, 1986). Significant electrophoretic studies on bacteria have generally shown that what is currently recognized as a single species does contain genetic diversity among strains at the 50% level or greater (Ochman *et al.*, 1983; Musser *et al.*, 1985; Selander *et al.*, 1985; Reardon *et al.*, 1986). Criteria for delineation of bacterial species based on allelic diversity remain largely subjective but further investigations of this nature may lead to a more objective approach.

Although the results presented here cannot conclusively identify the boundaries between species in the genus *Clavibacter*, they do have some useful comparative value. For instance, the *Clavibacter* sp. ex *Lolium*, which has been frequently called *C. rathayi* on the basis of inadequate identification (Riley, 1987), showed as much difference from *C. rathayi* as *C. rathayi* did from the other named species. Similarly, the cluster analysis would indicate that '*Corynebacterium agropyri*' was distinct from the named species. On this basis, it would be reasonable to conclude that both *Clavibacter* sp. ex *Lolium* and '*Corynebacterium agropyri*' are distinct species, with the distinction being somewhat clouded only by the variation within *Clavibacter michiganense*. However, a reasonable interpretation of the data presented here is that the three subspecies of *C. michiganense* should be considered distinct species.

Allozyme electrophoresis appears to be a useful tool for strain identification in bacteria. The duplicated strains from different collections (*Clavibacter tritici* strains CS4 and CS17; *C. rathayi* strains CS5 and CS15) gave identical allelic profiles. Such a comparison including ATCC 13659, which is supposed to be identical to CS5 and CS15, would be useful because ATCC 13659 has been shown to be atypical of *C. rathayi*. ATCC 13659 has cell wall peptidoglycans based on lysine rather than 2,4-diaminobutyric acid and differing lipid composition (Collins, 1983) as well as being clearly unrelated to other *C. rathayi* strains in the numerical taxonomic study of Seiler (1983). Application of allozyme electrophoresis would resolve this problem.

In most cases where the allelic profiles were identical, the strains had related origins. The electrotypes A1 was isolated from South Australia, A3 from Western Australia, E3 from New

Zealand and F2 from Iran. The only electrotpe with strains of diverse origin was F3, with CS16 from India and CS21 from Iran. The correlation of the genetic similarity within a region may indicate the genetic homogeneity of the bacterial populations in a region at the time the strains were isolated or that the strains were collected from a single or closely related sites. The latter is not true for the South Australian strains, which were from widely distributed sites. Allozymic analysis of larger numbers of strains within the range of a particular bacterial species could provide much data on the geographic origins of the species and its history of dispersal.

The difference between the South Australian and Western Australian strains of *Clavibacter* sp. ex *Lolium* is worth noting. Studies of the adhesion of these strains to the cuticles of various *Anguina* spp. (I. T. Riley & A. C. McKay, unpublished) showed patterns that clearly indicate the genetic divergence or different origins of the populations from these two geographically isolated regions. The genetic differences that have been observed suggest the possibility of ecological differences between populations of *Clavibacter* sp. ex *Lolium*. Such ecological diversity would have significance for the management of this toxigenic plant pathogen.

We thank the Australian Meat and Livestock Research and Development Corporation for financial support. J. Gooden and J. Birrell are thanked for their technical assistance. Advice from M. Adams and P. Baverstock during the preparation of the manuscript was appreciated. Importation of bacterial strains was done under permit from the Australian Quarantine Service.

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