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Genetic Characterization and Diversity of *Rathayibacter toxicus*

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ABSTRACT

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Rathayibacter toxicus is a nematode-vectored gram-positive bacterium responsible for a gumming disease of grasses and production of a highly potent animal and human toxin that is often fatal to livestock and has a history of occurring in unexpected circumstances. DNA of 22 strains of *R. toxicus* from Australia were characterized using amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE). AFLP analysis grouped the 22 strains into three genetic clusters that correspond to their geographic origin. The mean similarity between the three clusters was 85 to 86%. PFGE analysis generated three different banding patterns that enabled typing the strains into three genotypic groups corresponding to the same AFLP clusters. The similarity coefficient was 63 to 81% for *Xba*I and 79 to 84% for *Spe*I. AFLP and PFGE analyses exhibited an analogous level of discriminatory power and produced congruent results. PFGE analysis indicated that the *R. toxicus* genome was represented by a single linear chromosome, estimated to be 2.214 to 2.301 Mb. No plasmids were detected.

The bacterium *Rathayibacter toxicus* (41,44) is responsible for a gumming disease and ryegrass toxicity (10,32,41) resulting in an often fatal poisoning of livestock in Australia (26). Although ryegrass toxicity has been reported in South Africa (46), no cultures of *R. toxicus* originating outside Australia are available. It is likely that contaminated *Lolium rigidum* L. (annual ryegrass) seed imported from Australia was responsible for this possible introduction. *Rathayibacter* spp. have been identified in orchard grass (*Dactylis glomerata* L.) seed in Oregon (1) and several reports have suggested the presence of *R. toxicus* in chewing's fescue (*Festuca nigrescens* Lam.) seed in Oregon (26,37).

R. toxicus cells are gram-positive rods of coryneform morphology, and have a B2γ type cell wall peptidoglycan with 2,4-diaminobutyric acid, predominant menaquinones of the MK-10 type, phosphatidyglycerol and diphosphatidyglycerol as basic polar lipids, and a high G+C content of 67 mol% (41,44). Several species of *Anguina* (seed and leaf gall nematodes) are known to carry *R. toxicus* into the host plant, where it colonizes the inflorescence or the galls formed by the nematode (30,33,34,37–40). Glycolipid toxins, known as corynetoxins, are produced by the bacterium as the grass matures and becomes senescent (15). Toxin production is considered to be regulated by a bacteriophage that is associated with the bacterium (28,36). Animals that consume infected plants suffer a toxicosis characterized by episodic neurological symptoms, often leading to death (5,18). In Australia, more than 100,000 sheep and thousands of cattle die from this disease in some years (14). In Australia, *R. toxicus* most commonly is found in *L. rigidum* with *Anguina funesta* as a vector (30), and *Polypogon monspeliensis* (rabbit-foot grass, called annual beardgrass in Australia) and *Lachnagrostis filiformis* (syn. *Agrostis avenacea*, annual blowngrass) with an undescribed *Anguina* vector (6,15,16,27). Gumming disease symptoms occasionally occur in other grasses, which are not hosts of any known nematode vector. In ryegrass swards infested with *Anguina funesta*, it is assumed that the nematode may invade and introduce *R. toxicus* into other plants at a low frequency, which further indicates that the bacterium is not host specific and may be latent in some hosts (10,34,35). Novel host–vector combinations for *R. toxicus*, namely *Triticum aestivum* (wheat) with *A. tritici* (33) and *Ehrharta longiflora* (annual veldt grass) with *A. australis* (43), have been demonstrated experimentally. Apparently, *R. toxicus* is not vector specific to a particular species of *Anguina* (38) and *R. toxicus* potentially can colonize and produce toxin in a wide range of cereals and fodder grasses, including species consumed by humans (10). This makes *R. toxicus* a high-threat-risk foreign pathogen (14).

Little is known about genetic diversity of the organism. Typing of *R. toxicus* strains using serology and bacteriophage sensitivity have shown little variation among strains, and allozyme electrophoresis produced incomplete data (21,32,36). Molecular techniques are considered the most accurate means of fingerprinting microorganisms on a species and subspecies level. Amplified fragment length polymorphism (AFLP) is one of the most powerful discriminatory fingerprinting techniques that employs selective amplification of restriction fragments from a digest of total genomic DNA (48). The technique has been used to characterize and fingerprint a broad range of plant pathogenic bacteria. (11,19,31). Pulsed-field gel electrophoresis (PFGE) has been used for typing a number of phytopathogenic bacteria (8,9,12,17). This sensitive fingerprinting technique is based on DNA macro restriction fragment variation obtained from the whole bacterial genome after digestion with a "rare cutting" endonuclease, followed by electrophoresis in a pulsed field (25).

Our objective was to determine the genetic diversity among strains of *R. toxicus* and examine their relationship to the geographic origin of strains, host plant and year of isolation.

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MATERIALS AND METHODS

Source of strains, growth, and extraction of DNA. The 22 strains of *R. toxicus* investigated (Table 1) were maintained by monthly transfer on nutrient broth yeast extract (NBY) agar (47). For DNA extraction, the bacteria were grown on a rotary shaker in Luria-Bertani broth (24). Genomic DNA was isolated by the modified Marmur method, as described (20,45). Concentrations of DNA were determined with a spectrophotometer, standardized to 100 ng/µl, and stored at –20°C.

AFLP. To obtain evenly distributed bands in the size range of 50 to 700 kbp, several combinations of "long cutters" (*Apa*I, *Eco*RI, and *Hin*dIII) and "short cutters" (*Hpa*II, *Hpy*CH4IV, *Mse*I, and *Taq*I) were tested.

Several of the strains received from different collections had an identical origin (Table 1). These strains were included as a reproducibility control. Template DNA preparation for selective amplification was carried out according to Vos et al. (48) with slight modifications. Briefly, genomic DNA (100 ng) of each strain was digested with a combination of restriction endonucleases, and corresponding adaptors were ligated to the resulting restriction fragments. For selective amplification, 1 µl of a 10-fold-diluted ligation mixture was amplified with EcoRI+0 (5′-GAC TGC GTA CCA ATT C) and HpaII+A (5′-CGA TGA GTC CTC ACC GGA) primers in a GeneAmp PCR System 2700 (Applied Biosystems). EcoRI+0 primer was labeled with infrared fluorescent dye IRDye 700 (Li-Cor Inc, Lincoln, NE). Electrophoresis of the amplified products was performed on a 6.5% polyacrylamide gel using a Li-Cor Long ReadIR DNA Sequencer (model 4200). Image data were collected automatically and simultaneously recorded in a digital format during electrophoresis. The reproducibility of AFLP analysis was evaluated by comparing the fingerprints derived from control (duplicate) strains.

PFGE. Cultures were grown overnight in NBY broth, harvested, and embedded in 1% low-melting-point agarose. The agarose plugs were transferred into lysis solution (10 mM Tris, pH 8.0; 250 mM EDTA, pH 8.0; 1 M NaCl; 0.2% sodium deoxycholate; and 0.5% *N*-lauroylsarcosine) supplemented with lysozyme at 1 mg/ml and incubated at 37°C for 24 h. After decanting the lysis solution, plugs were rinsed twice with cold ESP buffer (250 mM EDTA, pH 9.5, and 1.0% *N*-lauroylsarcosine). Then, the agarose blocks were treated for 48 h at 53°C with 2 ml of ESP buffer containing proteinase K at 1 mg/ml. The plugs were stored at 4°C in ESP buffer. For digestion with restriction enzymes, the agarose blocks were washed four times (every wash at least 30 min) with 2 ml of Tris-EDTA buffer, and restriction reactions with endonucleases were performed according to the manufacturer's instructions (New England Biolabs, Beverly, MA). After incubation overnight at 37°C, the reaction was stopped with ESP buffer.

The DNA fragments were separated in a CHEF-DR II unit (Bio-Rad, Hercules, CA) in a 1% agarose gel. Electrophoresis conditions and running buffer were selected to resolve target DNA fragment size ranges (7).

The exact conditions used are described in the gel legends. The size of the *R. toxicus* genome was determined from the total value of all the *Pac*I-generated fragments. The chromosomes of *Hansenula wingei* (1.05 to 3.13 Mb) (Bio-Rad), yeast chromosome PFG Marker (225 to 1,900 kbp), and low-range PFG marker (2.03 to 194 kbp) (New England Biolabs) were used as molecular weight markers. Gels were stained with ethidium bromide at 0.5 mg/liter for 30 min, destained in water for 2 h, and then digital images were made with the ChemiDoc EQ System (Bio-Rad). To assess reproducibility of PFGE, at least three DNA preparations and gel runs for each strain were performed independently.

Data analysis. Quantity One software (Bio-Rad) was used for band analysis (fragment size and band intensity). A binary data set was generated for each strain based on absence or presence of bands. Analyses of phylogenetic relationships were done with Phylogenetic Analysis Using Parsimony (PAUP), version 4 (Sinauer Associates, Sunderland, MA), and the dendograms were constructed using the unweighted pair group method with averages (UPGMA).

RESULTS

AFLP. The *Eco*RI/*Hpa*II combination with a set of amplification primers with one selective nucleotide EcoRI+0/HpaII+A produced the most suitable banding patterns. Up to 55 to 65

TABLE 1. Origin of strains of *Rathayibacter toxicus* used in this study and their amplified fragment length polymorphism (AFLP) group

Group, laboratory no.[y]	Received as[z]	Original source	Host	Location and year isolated
A				
FH-138	CS28 (ICMP 6307)	I. Riley	*Lolium rigidum*	Western Australia, 1978
FH-81	CRK73dy dark	A. Payne	*L. rigidum*	Western Australia, 1973
FH-82	CRK73dy light	A. Payne	*L. rigidum*	Western Australia, 1973
FH-86	CRW1 dark	A. Payne	*L. rigidum*	Western Australia, 1974
FH-87	CRW1 light	A. Payne	*L. rigidum*	Western Australia, 1974
FH-88	CRW3	A. Payne	*L. rigidum*	Western Australia, 1980
FH-89	CRW3-160	A. Payne	*L. rigidum*	Western Australia, 1980
FH-128	Freshly isolated from galls	I. Riley	*L. rigidum*	Western Australia, 2001
FH-139	CS31	D. Chatel	*Phalaris* sp.	Western Australia, 1981
FH-140	CS3	I. Riley	*L. rigidum*	Western Australia, 1983
FH-142	CS32	D. Chatel	*Austrodanthonia caespitosa*	Western Australia, 1981
FH-144	CS29	D. Chatel	*L. rigidum*	Western Australia, 1981
FH-145	CS30	D. Chatel	*Avena sativa*	Western Australia, 1980
FH-146	CS100	I. Riley	*A. caespitosa*	Western Australia, 1991
B				
FH-137	CS14 (ICMP 9525)	ATCC 49908	*L. rigidum*	South Australia, 1983
FH-83	CRS2 dark	A. Payne	*L. rigidum*	South Australia, 1975
FH-85	CRS3	A. Payne	*L rigidum*	South Australia, 1975
FH-84	CRS2 light	A. Payne	*L. rigidum*	South Australia, 1975
FH-141	CS2	A. F. Bird	*L. rigidum*	South Australia, 1983
FH-147	CS33	I. Riley	*L. rigidum*	South Australia, 1984
FH-183	CS34 (ICMP 8527)	I. Riley	*L. rigidum*	South Australia, 1984
C				
FH-100	SE3	K. Ophel-Keller	*Polypogon monspeliensis*	South Australia, 1991

[y] AFLP grouping based upon EcoRI+0/HpaII+A analysis.
[z] ICMP = International Collection of Micro-organisms from Plants. Auckland, New Zealand.

evenly distributed bands were recorded, and 16 of them were polymorphic. The duplicate strains of *R. toxicus* produced highly homologous AFLP patterns and exhibited more than 95% homology (data not shown). Only slight variation in band intensity was observed.

Cluster analysis (Fig. 1; Table 2) assigned the 22 *R. toxicus* strains into three AFLP clusters, A, B, and C, with a linkage of 84.7 to 90.1%. The similarity coefficient for strains belonging to the same cluster varied from 2.9 to 5.2%. There was a distinct pattern of strain grouping according to their geographical origin. Cluster A included 14 strains, all from Western Australia and associated with *A. funesta*: FH-81, FH-82, FH-86, FH-87, FH-88, FH-89, FH-128, FH-138, FH-139, FH-140, FH-142, FH-144, FH-145, and FH-146. South Australian strains formed two clusters: cluster B contained seven strains all associated with *A. funesta*: FH-83, FH-84, FH-85, FH-137, FH-141, FH-147, and FH-183; and cluster C consisted of a single strain, FH-100, associated with *Anguina* spp. in *P. monspelienesis* from a geographically separate part of Australia.

PFGE. Genomic DNA isolated and purified in situ was digested with a number of restriction endonucleases. The choice of restriction enzyme for PFGE analysis was influenced by the high G+C content of *R. toxicus* (67 mol%) (44). Seven restriction endonucleases with A+T-rich recognition sites, such as 6-bp "cutters" *Ase*I (AT/TAAT), *Dra*I (TTT/AAA), *Eco*RI (G/AATTC), *Spe*I (A/CTAGT), *Ssp*I (AAT/ATT), and *Xba*I (T/CTAGA), and 8-bp cutter *Pac*I (TTAAT/TAA), were tested.

*Ase*I produced an extensive number of bands in the size range from 15 to 200 kbp. *Eco*RI and *Ssp*I generated too many fragments, all of them under 20 and 70 kbp, respectively. Digestion with *Dra*I resulted in 18 to 20 evenly distributed bands in a size range of 23 to 580 kbp. *Pac*I digestion resulted in 4 to 5 bands in a size range of 251 to 798 kbp (Fig. 2). *Spe*I and *Xba*I endonucleases produced the most suitable banding patterns for reliable PFGE analysis (Figs. 3 and 4).

PFGE exhibited a very high level of reproducibility; no variation between preparations of DNA or between runs was observed. Some bands were more intense or thicker, indicating closeness in

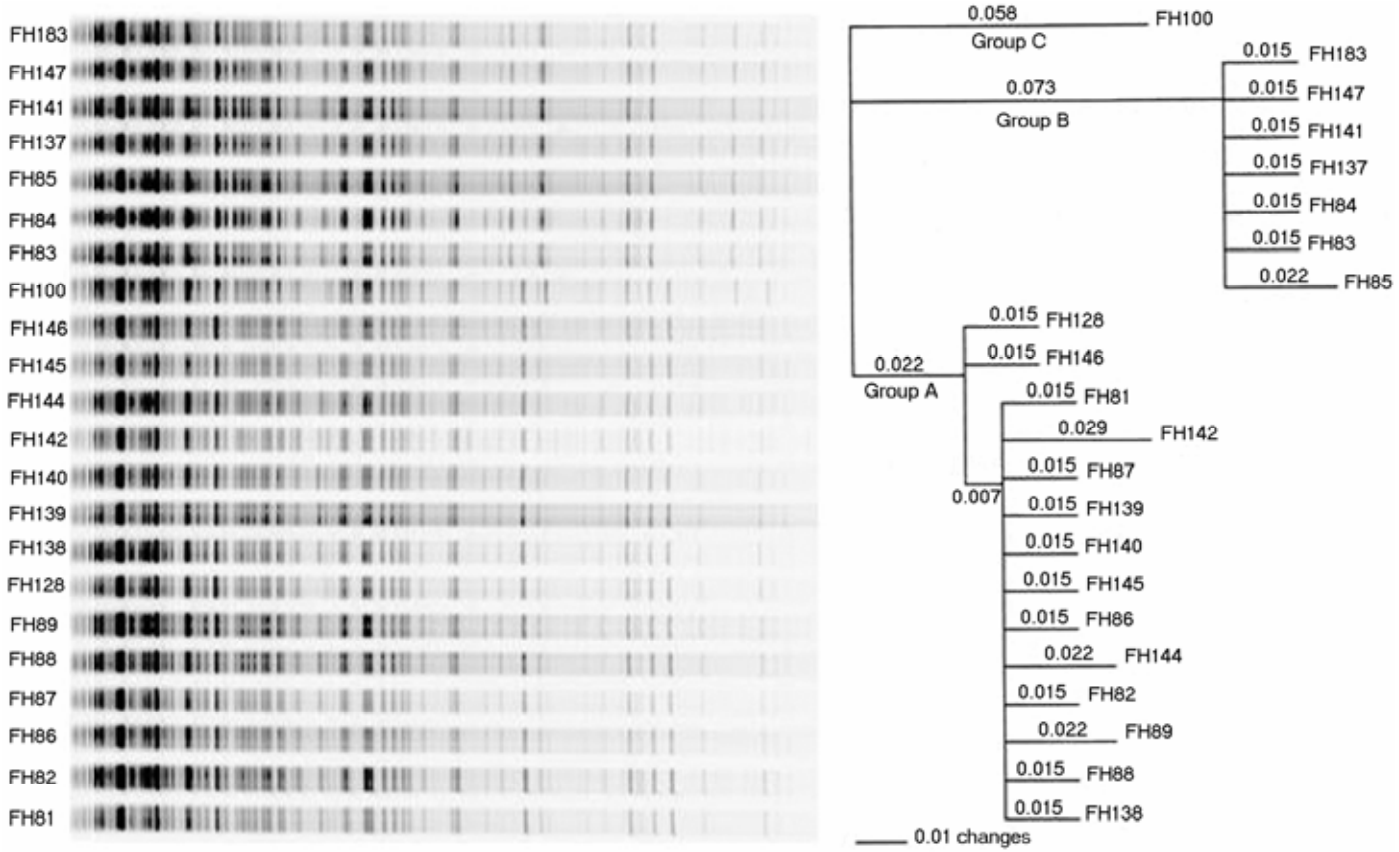

Fig. 1. Amplified fragment length polymorphism patterns based on *Eco*RI/*Mse*I digestion and phylogenetic tree of 22 strains of *Rathayibacter toxicus*. Analysis of phylogenetic relationships was done with Phylogenetic Analysis Using Parsimony (PAUP), version 4 (Sinauer Associates, Sunderland, MA). The dendrogram was constructed using the unweighted pair group method with averages. The bar indicates 1% divergence and branch length shows dissimilarity between strains.

TABLE 2. Similarity percent for pairwise comparison of genomic fingerprints of *Rathayibacter toxicus* strains

			Pulsed-field gel electrophoresis			
	Amplified fragment length polymorphism		*Spe*I		*Xba*I	
Group	A	B	A	B	A	B
A[x]	94.9–97.1	...	100	…	100	…
B[y]	84.7–87.6	96.4–97.1	84.2	100	81.3	100
C[z]	88.3–90.1	84.7–85.4	84.2	78.9	62.5	68.8

[x] Group of Western Australian strains: FH-81, FH-82, FH-86, FH-87, FH-88, FH-89, FH-128, FH-138, FH-139, FH-140, FH-142, FH-144, FH-145, and FH-146; all with *Anguina funesta* as a vector.
[y] Group of South Australian strains: FH-83, FH-84, FH-85, FH-137, FH-141, FH-147, and FH-183, all with *A. funesta* as a vector.
[z] Single strain (FH-100) from southeastern South Australia; vectored by *Anguina* spp. in the genus *Polypogon*.

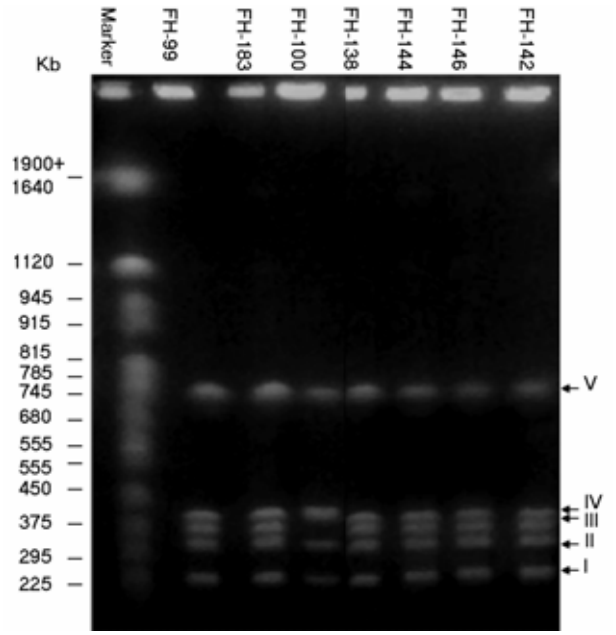

Fig. 2. Pulse-field gel electrophoresis pattern of *Pac*I-digested *Rathayibacter toxicus* strains. Electrophoresis conditions were 0.5× Tris-borate EDTA buffer, 200 V for 26 h, pulse time 70 s for 15 h, and then 120 s for 11 h. Marker was yeast chromosomes (New England Biolabs, Beverly, MA). The ladder size is shown on the left and the fragment designation is on the right.

size of bands that comigrate. For adequate resolution of all macrorestriction fragments, several gel runs with different pulse times were applied. The resultant separation of almost all of the restricted fragments did not increase the discrimination power of PFGE analysis. Therefore, we chose to use a single run with one pulse time change in the middle of the run. Fragments restricted with *Xba*I endonuclease produced 12 to 13 DNA fragments, depending upon the strain, with the majority of bands in the size range of 40 to 240 kbp (Fig. 3).

Digestion with *Spe*I generated 13 to 15 fragments in the range of 15 to 130 kbp (Fig. 4). Both enzymes enabled differentiating strains into three analogous haplotypes, similar to those observed by AFLP with similarity coefficients in the range of 78.9 to 84.2% for *Spe*I digests and 62.5 to 81.3% for *Xba*I (Table 2).

Genome organization and topology. Digestion of *R. toxicus* DNA from different geographical groups with *Pac*I endonucleases resulted in five fragments (I, II, III, IV, and V) in a size range of 251 to 798 kbp (Fig. 2; Table 3). The genome size of *R. toxicus* was estimated at 2.214 to 2.301 Mb.

Strain FH-100 exhibited four fragments in the same size range. The intensity of fragment III (448 kbp) was much stronger than that of fragments I (259 kbp) and II (351 kbp), indicating co-migration of two fragments that are very close in size. Analysis of band intensity with Quantity One (Bio-Rad) software showed the relative quantity (intensity expressed as percentage of the total intensity of all the bands in the lane) for fragment I at 19.3%, fragment II at 12.2%, fragment III at 40.2, and fragment IV at

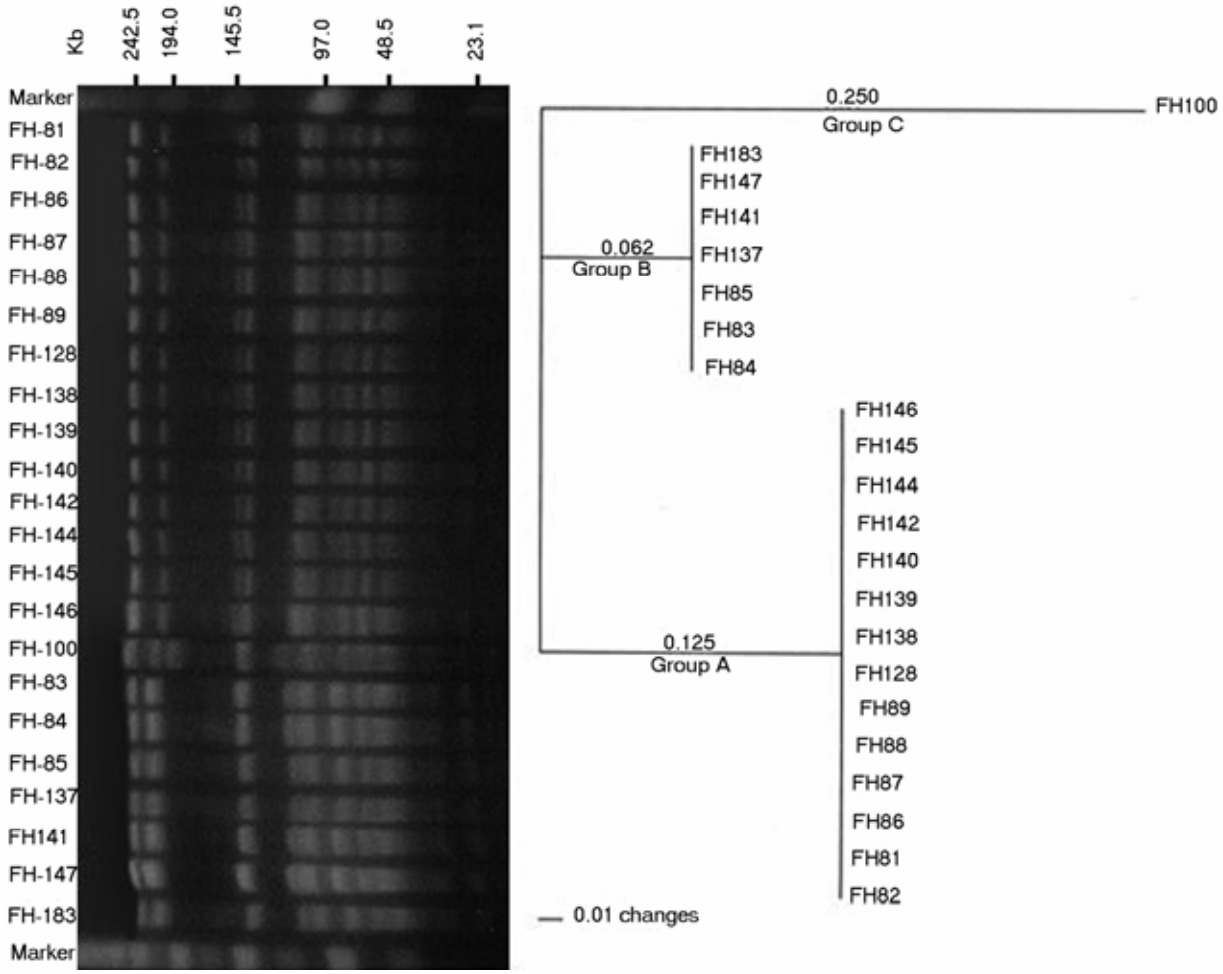

Fig. 3. Pulse-field gel electrophoresis (PFGE) patterns and phylogenetic tree of 22 strains of *Rathayibacter toxicus* digested with *Xba*I. Analysis of phylogenetic relationships was done with Phylogenetic Analysis Using Parsimony (PAUP), version 4 (Sinauer Associates, Sunderland, MA). The dendrogram was constructed using the unweighted pair group method with averages. The bar indicates 1% divergence and branch length shows dissimilarity between strains. The electrophoresis conditions consisted of the following: 0.5× Tris-borate EDTA buffer, 200 V with ramped pulses of 3 to 30 s for 8 h and of 1 to 13 s for 12 h. Marker: Low Range PFG Marker (New England Biolabs, Beverly, MA).

28.4%, thus confirming our prediction for the presence of two similar size fragments (448 kbp).

To avoid underestimation of the genome size and to demonstrate that *Pac*I restriction nucleases digested all genetic elements present in the *R. toxicus* genome, *R. toxicus* DNA (embedded into agarose blocks) was retrieved from the agarose gel wells, digested with *Eco*RI nucleases, and subjected to a second electrophoresis. No DNA fragments were detected, thus verifying that all genetic elements present in the *R. toxicus* genome were digested and resolved by *Pac*I analysis.

To investigate the presence of any extrachromosomal replicons in the *R. toxicus* strains, intact genome DNA in agarose blocks was subjected to PFGE for 60 h at 100 V with the pulse time ramped from 250 to 900 s. Results showed the megasized genomic DNA entered the gel and migrated as a distinct fragment size of 2.35 Mb (Fig. 5). To determine whether this band migrated as a linear chromosome in a pulse-time-dependent manner, the pulse conditions were changed to affect migration of only circular but not linear elements. The results showed the megasize fragment migrated as a linear DNA at a rate comparable with linear DNA ladders and consistently exhibited a size of 2.35 Mb.

To linearize any cryptic supercoiled circular elements possibly present in the *R. toxicus* genome, S1 nuclease treatment of genomic DNA in agarose plugs was performed (4). S1 nuclease digestion of *R. toxicus* DNA did not produce any additional bands and did not change the migration rate of the 2.35-Mb band of any strain.

DISCUSSION

The 22 Australian strains of *R. toxicus* were successfully fingerprinted using two DNA-based methods: AFLP and PFGE.

AFLP analysis (48) has proven to work well for fingerprinting bacteria at the strain level. It has been shown that 10 to 20 polymorphic bands are optimal for comparative analysis (22). The combination of enzymes and selective nucleotide extension of

TABLE 3. Size of *PacI*-digested fragments for three geographical groups (A, B, and C) of *Rathayibacter toxicus*

	Size (kbp)		
DNA fragment	A (FH-138)[x]	B (FH-99)[y]	C (FH-100)[z]
I	261	251	259
II	355	348	351
III	401	392	448
IV	432	429	448
V	798	794	795
Totals	2,247	2,214	2,301

[x] Group of Western Australian strains, with *Anguina funesta* as a vector.
[y] Group of South Australian strains, with *A. funesta* as a vector.
[z] A single strain, with *Anguina* spp. in genus *Polypogon* as a vector.

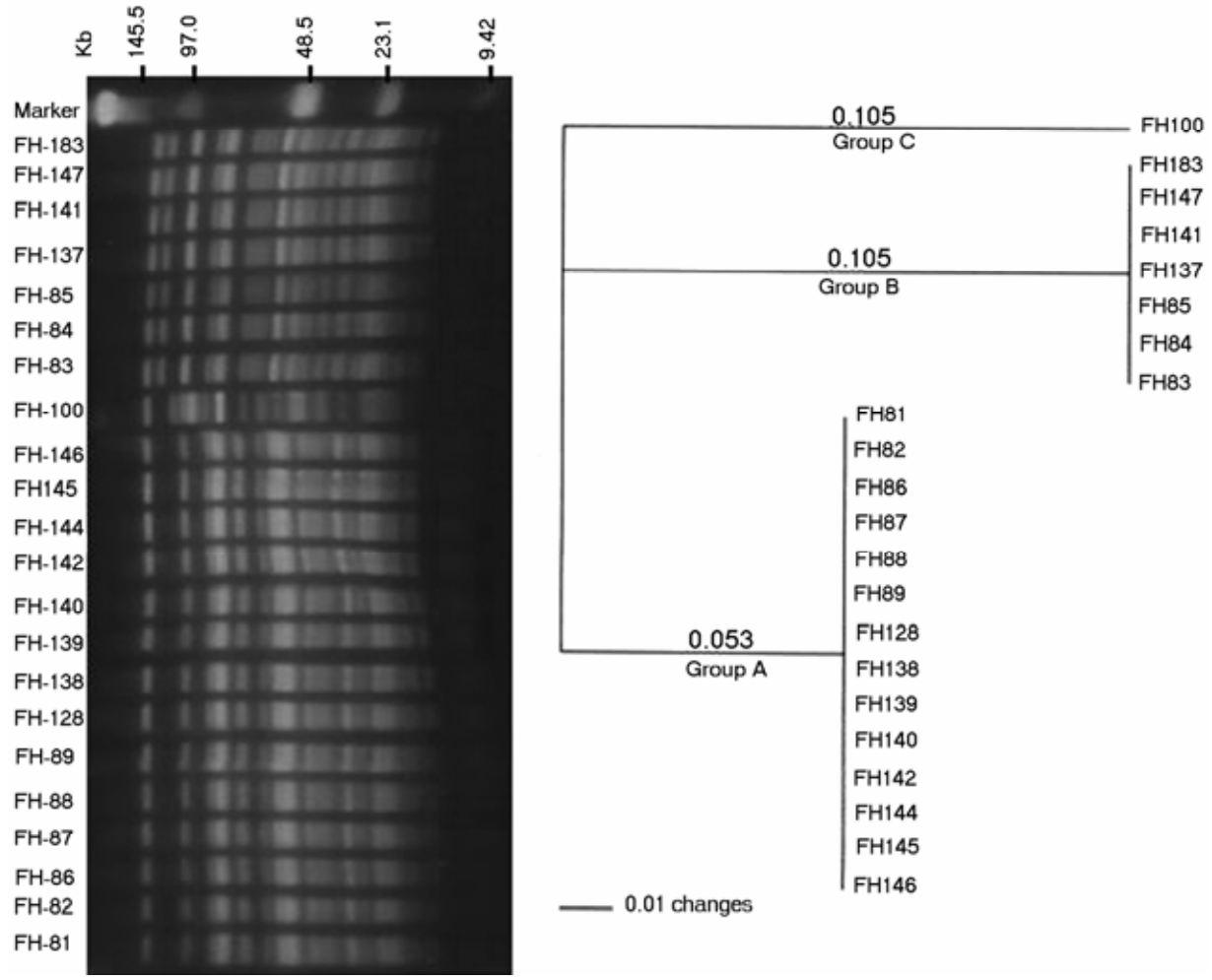

Fig. 4. Pulse-field gel electrophoresis (PFGE) patterns and phylogenetic tree of *Rathayibacter toxicus* digested with *Spe*I. Analysis of phylogenetic relationships was done with Phylogenetic Analysis Using Parsimony (PAUP), version 4 (Sinauer Associates, Sunderland, MA). The dendrogram was constructed using the unweighted pair group method with averages. The bar indicates 1% divergence and branch length shows dissimilarity between strains. The electrophoresis conditions consisted of the following: 0.5× Tris-borate EDTA buffer, 200 V with switch from 4 to 20 s for 4 h and from 1 to 10 s for 16 h. Marker: Low Range PFG Marker (New England Biolabs, Beverly, MA).

primers for AFLP analysis of *R. toxicus* were optimized; the EcoRI+0/HpaII+A primer combination produced a banding pattern suitable for fingerprinting of *R. toxicus* strains.

The results of these AFLP analyses were highly reproducible. The differences between control strains were less than 5%. Minor variability recorded between duplicate strains could arise from slight fluctuation within different AFLP reactions or the amount of polymerase chain reaction product loaded into the gel wells. The data show that up to 5% of the differences can be disregarded.

PFGE has been shown to be an effective diagnostic tool widely applied in clinical microbiology for bacterial strain identification and differentiation, and for fingerprinting and tracing sources of pathogenic organisms (25).

Macrorestriction digestion followed by electrophoresis in a pulsed field has been shown to be a valuable tool for differentiation of species and pathovars of many plant-pathogenic bacteria, such as closely related gram-positive *Clavibacter michiganensis* subsp. *sepedonicus* (8), *Curtobacterium flaccumfaciens* (17), and *Rhodococcus fascians* (29).

PFGE analysis using *Spe*I and *Xba*I restriction endonucleases enabled typing of 22 *Rathayibacter toxicus* strains into three geographical groups and showed a level of sensitivity similar to AFLP. The coherence of PFGE results from at least three independent experiments shows that PFGE analysis is a highly reproducible method for typing *R. toxicus* strains.

Application of *Pac*I restriction nuclease allowed digestion and resolution of the whole *R. toxicus* genome. A second digestion with *Eco*RI agarose plugs, recovered from gel wells after *Pac*I analysis, did not indicate the presence of any DNA left undigested by *Pac*I. The choice of *Eco*RI restriction endonuclease for a second digestion was influenced by the fact that this enzyme cuts *R. toxicus* DNA into fragments of less than 20 kbp, thus assuring that no DNA elements remained unresolved.

The very close similarity in the *R. toxicus* genome size obtained from intact DNA (2.35 Mb) and from *Pac*I-digested DNA (2.214 to 2.301 Mb) corroborates the conclusion that all genetic elements present in the bacterium were resolved. The 2.35-Mb replicon in *R. toxicus* appears to be linear and is the only chromosome.

The first linear bacterial chromosome was described in 1989 for *Borrelia burgdorferi* (3). Since then, linear chromosomes have been described for several *Streptomyces* spp. (23,49) and the gram-positive bacterium *Rhodococcus fascians* strain D188 (13). However, the latter report was disproved by Pisabarro et al. (29). The genome of the gram-negative *Agrobacterium tumefaciens* C58 includes four replicons: two plasmids and two chromosomes, a circular one of 3.0 Mb and a linear one of 2.1 Mb (2). Thus, our description of a linear chromosome in *R. toxicus* is unusual but not unique for a bacterial genome.

The *R. toxicus* genome is 2.214 to 2.301 Mb. This is similar in size to the 2.50 to 2.64 Mb for the related gram-positive plant pathogenic *Clavibacter michiganensis* subsp. *sepedonicus* (8).

A number of genotypic methods, including serology, multilocus enzyme electrophoresis (MEE), and phage typing have been investigated for typing *R. toxicus* strains (21,32,36,42). The serological analysis and phage typing failed to reveal any differences among strains of *R. toxicus*. Population genetic analysis using MEE resulted in contradictory results. Johnson et al. (21) separated 52 strains of *R. toxicus* into 12 groups with a genetic distance of 0.06 to 0.23; there was no clear pattern of strain distribution with respect to evolutionary time, place, or host plant of origin. In contrast, Riley et al. (42), using allozyme electrophoresis, typed 11 strains of *R. toxicus* into three MEE clusters with a linkage of 82%. The clusters corresponded very well with their geographical origin. Cluster A1 included strains CS1 (FH-188), CS2 (FH-141), CS14 (FH-79), and CS34 (FH-183) from South Australia; cluster A2 consisted of strain CS33 (FH-147) only; and cluster A3 included strains CS3 (FH-140), CS28 (FH-138), and CS32 (FH-142) from Western Australia. Based on MEE and differences in the specificity of the adhesion of these strains to the cuticle of their nematode (*Anguina* spp.) vectors, Riley and McKay (38) proposed that there was a genetic divergence among the populations from these two geographically isolated regions.

Our results agree with the results of Riley et al. (42) except for results of strain CS33 (FH-147). They typed this strain into MEE cluster A2, whereas we found it typed with the majority of the other South Australian strains (group B) with a linkage of 96.4 to 97.1% by AFLP typing and 100% by PFGE typing.

We analyzed 22 representative strains of *R. toxicus*. The strains were isolated from a broad range of host plants of different geographical regions within Australia over a period of more than 30 years. The genome of *R. toxicus* seems to be highly conserved, with high similarity values for both AFLP (84.7 to 97.1%) and PFGE (62.5 to 100%). The AFLP and PFGE data showed that the population of *R. toxicus* is very stable and has not undergone changes over many years. West Australian strains FH-81, FH-82, FH-86, FH-87, and FH-138 isolated in the 1970s were indistinguishable from strain FH-128 isolated 31 years later in 2001. The population structure was not host plant specific. Strains isolated from *L. rigidum* (FH-81, FH-82, FH-86, FH-87, FH-88, FH-89, FH-128, FH-138, FH-140, and FH-144) exhibited band patterns similar to strains isolated from *Phalaris* spp. (FH-139), *Austrodanthonia caespitosa* (FH-142, FH-146), and *A. sativa* (FH-145 and FH-146). In all cases, these infected plants were found among infected *L. rigidum* and represent incidental, nonspecific invasion by *Anguina funesta* of grasses in which it is unable to reproduce (40). Apparently, *R. toxicus* is not host specific and its infection of grass is determined largely by the specificity of its vector nematode.

Not only is there no evidence of genetic change over the period that the *R. toxicus* strains were collected, but the lack of genetic variation within the two geographical regions, separated by 1,800 km, also suggests that these areas are not within the bacterium's center of origin. Given that *L. rigidum* is an exotic weed of cropping regions in Western and South Australia, it is likely that *A. funesta* also was introduced. The center of origin of the

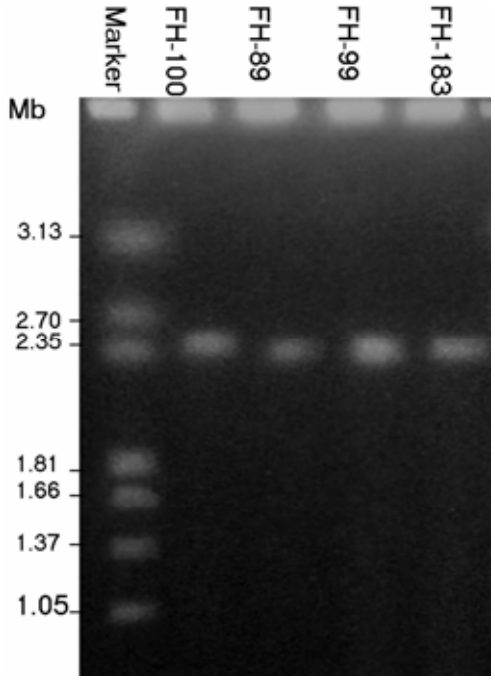

Fig. 5. Pulse-field gel electrophoresis of intact *Rathayibacter toxicus* DNA. Electrophoresis conditions were 1× Tris-acetate EDTA buffer, 100 V for 60 h, pulse ramped from 250 to 900 s. Marker was chromosomes of *Hansenula wingei* (Bio-Rad) with the ladder size shown on the left.

bacterium is not known; however, the finding that *R. toxicus* from *P. monspeliensis* is distinct indicates a need for further characterization of strains associated with this vector in *P. monspeliensis* and *Lachnagrostis filiformis*. *L. filiformis* is an Australian native and its populations are geographically separated from the area of *A. funesta* infestation in *Lolium rigidum*; therefore, it could be the original source of the bacterium. However, the conjectured association of corynetoxins with a different nematode in *F. nigrescens* in the United States and New Zealand (37) suggests that the center of origin of *R. toxicus* may not have been Australia despite its common occurrence as an economic pest in that country.

Despite apparent differences between AFLP and PFGE analyses, the techniques generally have similar approaches to fragment generation. They both employ total genomic DNA and rely on the presence or absence of specific restriction sites along the whole bacterial genome. In both techniques, the number and size range of fragments can be adjusted by a choice of restriction enzymes. PFGE reveals all macro restriction fragments derived from the genome and enables one to obtain information about genome topology and complexity of organization. In contrast, AFLP does not provide any data on genome organization and generates only a subset of amplified fragments. However, AFLP is faster to perform, less laborious, does not require expensive equipment, and is more applicable for a large number of strains tested simultaneously.

Most molecular-based typing techniques employ restriction fragments derived from a gene or a single chromosome locus. An accidental recombination (rearrangement, insertions, deletions, or point mutation) might influence the genotypic results derived from a single locus. The advantage of AFLP and PFGE over such techniques is that multiple bands are derived from the entire genome, increasing their reliability.

The comparative analysis of data obtained with PFGE and AFLP showed that both methods produce congruent results, thus having similar levels of reliability. The only divergence between data produced by PFGE and AFLP analyses was a different linkage level among strains. The higher AFLP similarity coefficients compared with those obtained with PFGE most likely are due to the large number of generated fragments available for statistical analysis.

Results of AFLP and PFGE reveal the presence of three distinct haplotypes among the 22 strains of *R. toxicus* from Australia. These results show that AFLP and PFGE analyses are both robust and reproducible and provide a high level of discrimination among strains of *R. toxicus.*

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