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Genetic analysis of *Clavibacter toxicus*, the agent of annual ryegrass toxicity

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SUMMARY

Multilocus enzyme electrophoresis was used to examine the relatedness of 52 isolates of *Clavibacter toxicus*, the agent of annual ryegrass toxicity. These included 37 Western Australian (WA) field isolates sampled in 3 distinct locations over a 2-year period, and 15 isolates sampled from 6 different host plant species in 3 states in Australia over approximately 8 years. Seventeen reference strains for the related genera *Curtobacterium*, *Rhodococcus* and *Arthrobacter* were examined for comparison. The 69 isolates were divided into 29 electrophoretic types (ETs), separated by genetic distances of 0·06 to 0·81. The *C. toxicus* isolates fell into 12 ETs, 11 of which formed a tightly clustered group separated by a genetic distance of 0·23 or less. Thirty-one of the WA field isolates of *C. toxicus* fell into a single ET, and four into another ET. *Clavibacter toxicus* therefore formed a closely related group which was genetically distinct from the other plant pathogenic species, and a dominant widely disseminated strain of the species was identified in WA.

INTRODUCTION

Clavibacter toxicus is the causative agent of the corynetoxin poisonings annual ryegrass toxicity (ARGT) and flood plain staggers. The bacterium is carried onto the host plants by *Anguina* spp. nematodes, where they infect the forming seeds and produce corynetoxins that are poisonous to livestock that graze the grass [1]. ARGT occurs as a major problem in Western Australia (WA) and South Australia (SA) where livestock graze *Lolium rigidum* (annual ryegrass) that is infected with *C. toxicus* [2–4]. Flood plain staggers occurs in SA where the grass *Polypogon monspeliensis* (annual beardgrass) is infected by *C. toxicus*, and in New South Wales (NSW) where *Agrostis avenacea* (blown grass) is infected [1, 5, 6]. ARGT has also been recorded in South Africa [7]. Both diseases primarily affect sheep, but cattle, horses and goats also can be affected and killed [7, 8]. ARGT is the most serious of the corynetoxin poisonings in terms of livestock mortalities and morbidities, especially in WA, where the disease has spread widely [1, 9, 10].

The plant host range of the bacteria was thought to be limited to *Lolium rigidum*, *Agrostis avenacea* and *Polypogon monspeliensis*, but *C. toxicus* also has been seen occurring naturally on *Avena fatua* (wild oats), *Avena sativa* (cultivated oats), *Danthonia caespitosa* (winged wallaby grass) and *Phalaris* sp. (canary grass), but usually only in densely infected paddocks of *L. rigidum*, where the nematode accidentally carries the bacteria onto the plant [11, 12].

Clavibacter toxicus shows differences from other *Clavibacter* spp., and within-species variation also has been recorded, based on parameters such as bio-

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chemical and physiological indicators [12, 13], serological properties [14], genetic grouping by multilocus enzyme electrophoresis (MEE) analysis [5, 15], bacteriophage sensitivity [16], and adhesion to the *Anguina* nematode [17]. An MEE study by Riley and colleagues showed that *C. toxicus* shared approximately 15% genetic similarity with other *Clavibacter* spp., whilst 8 isolates of *C. toxicus* from SA and WA were divided into 3 electrophoretic types, with 5 and 18% variation between them respectively [15]. A later MEE study, using six *C. toxicus* strains, including those isolated from *A. avenacea* and *P. monspeliensis*, showed a 10–20% variation amongst the organisms [5].

MEE is a method for genetic characterization of bacterial populations, based on the analysis of electrophoretic mobilities of constitutive enzymes extracted from the bacteria. It can be used for differentiation at the species and sub-species level [18]. The rationale behind this technique is that variations in electrophoretic mobility of enzymes have a genetic basis. Electrophoretic mobilities of enzymes are determined by their nett electrostatic charge, hence the rate of migration of an enzyme in a gel is related to its animo acid sequence, and therefore its gene locus [19].

The purpose of this study was to use MEE to examine the extent of genetic variation amongst a large population of *C. toxicus* field isolates from Western Australia. For comparative purposes other Australian strains of *C. toxicus*, and reference strains for related genera, both of which previously had been analysed [5, 15], were included in the examination. It was intended to relate genetic variation to the distribution and diversity of *C. toxicus* isolates in WA, where ARGT is an important condition which limits production of sheep.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Clavibacter toxicus and members of 12 related bacterial species or subspecies, represented by 69 strains or field isolates, were examined (Table 1). These included 37 WA field isolates of *C. toxicus*, all sampled from *L. rigidum* over a 2-year period in three distinct geographical areas in the ARGT zone of southwest WA. The northern sampling region included the shires of Dalwallinu and Wongan/Ballidu, the central region the shires of Cunderdin, Tammin, and Kellerberrin, and the southern region the shires of Wickepin, Kulin and Dumbleyung. Thirteen of the 37 field isolates were sampled in the northern region, 8 in the central region and 16 in the southern region. Nineteen of the 37 field isolates were sampled during the summer of 1990/1 (6 northern isolates, 3 central isolates, and 10 southern isolates) and 18 were sampled in the summer of 1991/2 (7 northern isolates, 5 central isolates, and 6 southern isolates). Single colonies from primary cultures of field isolates were subcultured onto 523M agar to ensure purity, and all cultures were grown and maintained on 523M agar [13]. Fifteen reference strains of *C. toxicus* isolated from WA, SA and NSW, sampled from six host plant species, *Lolium rigidum*, *Agrostis avenacea*, *Polypogon monspeliensis*, *Avena sativa*, *Danthonia caespitosa*, and *Phalaris* sp. also were analysed (Table 1). The remaining 17 reference strains belonged to the species *Rhodococcus fascians* (1 strain), *Arthrobacter ilicis* (1 strain), *Curtobacterium flaccumfaciens* (4 strains), *Clavibacter michiganense* (2 strains), *Clavibacter tritici* (2 strains), *C. iranicum* (1 strain), '*Corynebacterium agropyri*' (1 strain) and *C. rathayi* (5 strains). The reference strains were obtained from Dr I. T. Riley, and had the same CS identification numbers described in previous publications [13–17, 20, 21]. They were used under conditions approved by Australian Quarantine and Inspection Service.

Preparation of cellular enzymes

Isolates were streaked from pure cultures onto between 3–5 523M plates and incubated in the dark at room temperature for 7–10 days; the agar plates were sealed to prevent dehydration of the agar. Approximately 10^{11} cells were required per isolate for sufficient enzymes for MEE analysis [19]. Cells were harvested from the agar plates, washed in sterile phosphate buffered saline (pH 7·2) and centrifuged at 15000 g for 20 min. The cells were suspended in 1 ml 10 mM Tris–1 mM EDTA–0·5 mM NADP (pH 6·8), sonicated on ice for eight cycles of 1 min each, using a 100 W sonic probe (Braunsonic 1510), and after centrifugation at 20000 g for 20 min the supernatants were transferred into microcentrifuge tubes in 150 μl aliquots, and stored at −70 °C.

Multilocus enzyme electrophoresis

The supernatants were subjected to electrophoresis in horizontal 13·8% starch gels, and the electrophoretic

Table 1. *Identification and origin of bacterial reference (CS) strains analysed by MEE*

CS no.	Species	Other ID no.	Origin
1	*Clavibacter toxicus*	—	ex. *Lolium rigidum*, SA
2	*Clavibacter toxicus*	—	ex. *L. rigidum*, SA
3	*Clavibacter toxicus*	—	ex. *L. rigidum*, WA
5	*Clavibacter rathayi*	ICMP 2571	—
7	*Clavibacter rathayi*	ICMP 2573	—
8	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	ICMP 2584	—
9	*Curtobacterium flaccumfaciens* pv. *beta*	ICMP 2594	—
10	*Arthrobacter ilicis*[TS]	ICMP* 2607	—
13	*Clavibacter iranicum*[TS]	ICMP 3497	—
		NCPPB† 2253	—
14	*Clavibacter toxicus*	—	ex. *L. rigidum*, SA
15	*Clavibacter rathayi*	NCPPB 80	—
16	*Clavibacter tritici*	NCPPB 471	—
		ICMP 2627	—
18	*Clavibacter rathayi*	NCPPB 797	—
19	*Curtobacterium flaccumfaciens* pv. *poinsettiae*[TS]	NCPPB 854	—
21	*Clavibacter tritici*[TS]	NCPPB 1857	—
		ATCC‡ 11403	—
23	*Curtobacterium flaccumfaciens* pv. *oortii*	NCPPB 2113	—
		ATCC 25283	—
24	*Clavibacter michiganense* subsp. *nebrakense*[TS]	NCPPB 2581	—
		ATCC 27794	—
25	*Clavibacter michiganense* subsp. *michiganense*[TS]	NCPPB 2979	—
26	*Clavibacter rathayi*[TS]	NCPPB 2980	—
27	*Rhodococcus fascians*[TS]	NCPPB 3067	—
28	*Clavibacter toxicus*	WSM¶ 188	ex. *L. rigidum*, WA
29	*Clavibacter toxicus*	WSM 454	ex. *L. rigidum*, WA
30	*Clavibacter toxicus*	WSM 447	ex. *Avena sativa*, WA
31	*Clavibacter toxicus*	WSM 455	ex. *Phalaris* sp., WA
32	*Clavibacter toxicus*	WSM 456	ex. *Danthonia caespitosa*, WA
33	*Clavibacter toxicus*	—	ex. *L. rigidum*, SA
34	*Clavibacter toxicus*	—	ex. *L. rigidum*, SA
35	*Corynebacterium agropyri*	CA-1§	—
40	*Clavibacter toxicus*	—	ex. *Agrostis avenacea*, NSW
41	*Clavibacter toxicus*	—	ex. *Polypogon monspeliensis*, SA
100	*Clavibacter toxicus*	—	ex. *D. caespitosa*, WA
NSW	*Clavibacter toxicus*	—	ex. *A. avenacea*, NSW

[TS] Type strain or pathogen type.
* International Collection of microorganisms from Plants, Auckland, New Zealand.
† National Collection of Plant Pathogenic Bacteria, Harpenden, UK.
‡ American Type Culture Collection, Rockville, Maryland, USA.
¶ Western Australian Department of Agriculture Culture Collection.
§ See Murray [26].

mobilities of the following 17 enzymes were determined by staining for their specific enzyme activity, as recommended [19]: glyceraldehyde-3-phosphate dehydrogenase (G3PD) and nucleoside phosphorylase (NP) assayed in Tris–maleate buffer (pH 8·2); alanine dehydrogenase (ALA), catalase (CAT), fructose-1,6-diphosphatase (FDP), L-leucyl-tyrosine peptidase 1 (LT1), and 6-phosphogluconate dehydrogenase (6PGD) assayed in Tris–citrate buffer (pH 8·0); adenosine deaminase (ADA), hexokinase 1 and 2 (HEX1 and HEX2), isocitrate dehydrogenase (IDH), mannose-6-phosphate isomerase (MPI), L-leucyl-glycylglycine peptidase (LGG), L-leucyl-proline peptidase (LP), L-leucyl-tyrosine peptidase 2 (LT2) and phosphoglucomutase 1 and 2 (PGM1 and PGM2) assayed in lithium hydroxide buffer.

Distinctive mobility variants for each enzyme were numbered in order of decreasing anodal migration,

and were interpreted as products of different alleles at the corresponding structural gene locus. For each isolate, the allelic form for each enzyme was determined at least twice, and extracts from all isolates showing the same allele for an enzyme were run together on the same gel to confirm they were the same.

The genetic diversity at each enzyme locus was calculated from the formula $h = 1 - \Sigma x_i^2 \{n/(n-1)\}$, where x_i is the frequency of the ith allele at the locus, n is the number of isolates and $n-1$ is a correction for bias in small samples [22]. The mean number of alleles per enzyme locus was calculated for all 69 isolates, and also specifically for the *C. toxicus* isolates. Isolates with the same allele profiles over the 17 loci were assigned to the same electrophoretic type (ET). Genetic distance between ETs was calculated as the proportion of loci at which dissimilar alleles occurred. This was calculated using a computer clustering programme, Phentree, which is based on the TAXAN2 package, and which also used the resultant matrix of genetic distances to create a phenogram using the unweighted pair-group method of arithmetic averages (UPGMA) clustering fusion strategy [23, 24]. A bootstrap analysis was then performed to test the robustness of the groupings obtained. The Phylip package [25] was used to perform bootstrapping on the raw data (100 sets of the same number of random loci with replacement) followed by calculation of Nei's genetic distance and UPGMA clustering. The analysis was performed on a subset of the data represented by ETs 9–29, which included *Corynebacterium agropyri* and all the *Clavibacter* isolates other than the distinct *C. michiganense*.

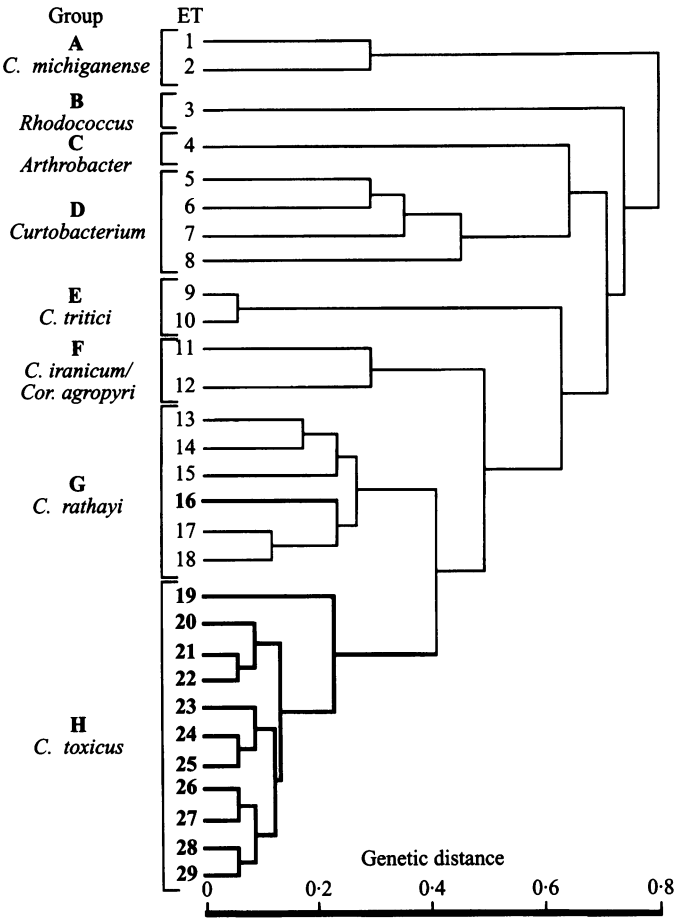

Fig. 1. Phenogram of genetic distances amongst 29 ETs of plant pathogenic bacteria, clustered by UPGMA strategy. Numbering of ETs is described in the text and in Table 2. ETs outlined in bold contain *C. toxicus* isolates.

RESULTS

Enzyme activities and genetic diversities

Sixteen of the 17 loci were polymorphic for the 69 isolates examined (HEX2 was monomorphic), whilst among the 51 *C. toxicus* CS reference strains and WA field isolates, only seven were polymorphic (LGG, ALA, LT2, ADA, G3PD, LT1, and MPI). Four null alleles where no enzyme activity was observed on the stained starch gel were recorded, at the following loci: ADA for strain CS 27 and one field isolate; PGM1 for strain CS 24; G3PD for strain CS 10.

A mean of 4·29 alleles per locus and a mean genetic diversity per locus of 0·29 were observed for the 69 isolates. The 52 *C. toxicus* isolates were located in 12 ETs. Eleven of these were closely related, but ET 16, containing isolate CS 3, clustered with *C. rathayi* strains. The mean genetic diversity amongst the remaining 51 *C. toxicus* isolates was 0·04, with the mean number of alleles per locus being 1·53.

Genetic distance between clusters

A phenogram was drawn from the matrix of genetic distance coefficients (Fig. 1). The 69 isolates were located in 29 ETs, divided into eight major groups identified as A–H, separated by genetic distances of between 0·29–0·81. All but two groups contained only one species, these exceptions being cluster F which contained *C. iranicum* (CS 13) and '*Corynebacterium agropyri*' (CS 35), and cluster G which contained all five *C. rathayi* isolates and the *C. toxicus* reference strain CS 3. The genetic variation observed between isolates from the same species ranged from 0·06–0·43.

The two *C. michiganense* strains contained in ETs 1 and 2 within cluster A were separated from the rest of the phenogram by a genetic distance of 0·8. Clusters B

Table 2. *Distribution of* C. toxicus *isolates into their electrophoretic types*

ET	Isolate	State	Host plant
19	CS 40	NSW	*Agrostis avenacea*
20	CS 33	SA	*Lolium rigidum*
21	CS 30	WA	*Avena sativa*
	CS 31	WA	*Phalaris* sp.
	CS 32	WA	*Danthonia caespitosa*
	NSW	NSW	*A. avenacea*
	Four field isolates	WA	*L. rigidum*
22	CS 14	SA	*L. rigidum*
	CS 34	SA	*L. rigidum*
23	CS 100	WA	*D. caespitosa*
24	CS 29	WA	*L. rigidum*
25	CS 41	SA	*Polypogon monspeliensis*
26	CS 28	WA	*L. rigidum*
	31 field isolates	WA	*L. rigidum*
27	CS 1	SA	*L. rigidum*
	CS 2	SA	*L. rigidum*
28	1 field isolate	WA	*L. rigidum*
29	1 field isolate	WA	*L. rigidum*

CS 3 has been omitted because of its clustering with *C. rathayi*.

and C were formed by single ETs 3 and 4, containing strains of *Rhodococcus fascians* (CS 27) and *Arthrobacter ilicis* (CS 10) respectively. Cluster D contained the four *Curtobacterium flaccumfaciens* pathovars in four ETs (ETs 5–8). This species was the most genetically diverse, with genetic distances between ETs from 0·18–0·43. The two *C. tritici* strains in cluster E (CS 16 and CS 21) fell into ET 9 and ET 10, separated by a genetic distance of 0·06. Cluster F contained two species, *C. iranicum* (CS 13) in ET 11, and *Corynebacterium agropyri* (CS 35) in ET 12, separated by a genetic distance of 0·29. Cluster G contained the five *C. rathayi* isolates, in ETs 13–15 and 17–18, as well as the *C. toxicus* reference strain CS 3, in ET 16. Genetic distances between these ETs were 0·2–0·27.

Genetic distances between *C. toxicus* isolates

Cluster H contained 51 of the 52 *C. toxicus* isolates examined, grouped into ETs 19–29 (Table 2). Genetic distances between these 11 ETs were 0·06–0·23. The seven CS *C. toxicus* strains isolated from *L. rigidum*, 3 of which were from WA and 4 from SA, fell into 5 ETs which were separated by genetic distances from 0·06–0·14. The SA isolates from *L. rigidum* were in different ETs from the WA isolates from this plant.

Table 3. *Bootstrap values for groups of plant pathogens*

Group	No. of ETs	No. of isolates	Bootstrap value (%)
C. tritici	2	2	96
C. iranicum/ *Cory. agropyri*	2	3	93
C. toxicus (less CS 3)	11	52	62
C. rathayi (with CS 3)	6	6	55

The two NSW isolates from *A. avenacea* (associated with flood plain staggers) fell into separate ETs. One ET (ET 19) was quite distinct from the rest of the *C. toxicus* cluster, at a genetic distance of 0·23, but the other isolate (CS NSW) fell into ET 21, which contained seven other WA isolates. ET 25 contained CS 41, isolated from a SA outbreak of flood plain staggers where *P. monspeliensis* was the host plant, and was separated from ET 24, containing CS 29, a strain isolated from *L. rigidum* in WA, by a genetic distance of 0·06. The final four strains, isolated from WA hosts other than *L. rigidum*, and therefore likely to be 'accidental infections', were separated into two ETs. ET 21 contained three of the samples; CS 30 isolated from *A. sativa*, CS 31 isolated from *Phalaris* sp., and CS 32 isolated from *D. caespitosa*. The fourth WA CS strain (CS 100), also isolated from *D. caespitosa*, was in ET 23, separated from ET 21 by a genetic distance of 0·14.

The 37 WA field isolates of *C. toxicus* fell into four ETs. ET 26 contained 31 of the 37 isolates (84%); ten from the northern sampling region, 7 from the central and 14 from the south. In addition 16 of the 31 isolates in this ET were sampled in the 1990/1 ARGT season and 15 from the 1991/2 season. ET 21 contained another four of the field isolates, and was separated from ET 26 by a genetic distance of 0·14. Two of these isolates were from the northern sampling region, and one each from the central and southern regions. Half were from the 1990/1 season. ETs 28 and 29 each contained a northern 1990/1 isolate, and were separated from each other by a distance of 0·06, from ET 26 by a distance of 0·09, and from ET 21 by 0·14.

Bootstrap analysis

Isolates in ETs 9–29 were divided into four groups (i.e. *C. tritici*; *C. iranicum*/*Corynebacterium agropyri*;

C. toxicus (excluding CS 3); *C. rathayi* (including CS 3). Their bootstrap values, which correspond to the number of times that group appeared in 100 bootstrap replicates, are shown in Table 3. All groups were distinct.

DISCUSSION

Comparison of the grouping of the CS reference strains, in groups A–G, with the respective groupings in previous studies [5, 15] showed considerable differences in genetic distances between and within species. Generally, the reference strains were less genetically distinct from each other than previously reported, suggesting that future MEE studies of this group of plant bacteria should include more reference strains for this species. This may also help clarify the observation that *C. iranicum* (CS 13) clustered with '*Corynebacterium agropyri*' (CS 35), whilst in other studies these were clearly identified as distinct species [15, 17, 26]. The grouping reported here was supported by the results of bootstrap analysis of the data. The suggestion of Riley and colleagues [15] that the three subspecies of *C. michiganense* be considered as separate species of the genus *Clavibacter* also was not supported here. Riley's group noted a genetic distance of 0·45 between two of the subspecies, while in this study the same strains were only separated by a genetic distance of 0·176. The morphology of the two strains in culture however differed considerably from each other, and from the other *Clavibacter* spp. (results not shown). This also was the most genetically distinct group from the other *Clavibacter* spp. (Group A, Fig. 1). The taxonomy of *C. michiganense* therefore is unclear, which is unfortunate since it is the type species for the genus *Clavibacter* [27].

Curtobacterium flaccumfaciens was shown to be genetically distinct from other plant pathogenic coryneforms, and contained four genetically diverse pathovars, which possibly should be considered as separate species in the genus. These pathovars once were considered separate species of the genus *Corynebacterium*, but were renamed *Curtobacterium flaccumfaciens* in the early 1980s [28].

Fourteen of the *C. toxicus* CS reference strains fell into nine closely related ETs. There was no clear pattern of distribution of specific strains with respect to time or location of sampling, or host plant of origin because of the tight clustering of the group (Table 2). This tight clustering is consistent with the isolates representing one or few clonal groups. Clonality can be inferred when bacteria that are isolated from different geographical locations or at different times are found to be phenotypically and genetically similar, so that the likely explanation is a common origin or ancestor [29].

An interesting result was the clustering of a *C. toxicus* reference strain with the five *C. rathayi* strains. The validity of this clustering using the UPGMA strategy was supported by the results of the bootstrap analysis. When the bacterial pathogen of annual ryegrass was first recognized it was misidentified as *C. rathayi* [30, 31], and only later named *C. toxicus* [13]. All other *C. toxicus* isolates examined in this study differed from the *C. rathayi* cluster by a genetic distance of 0·41. The isolate concerned, CS 3, was originally recovered in Katanning in WA in 1983, and was transferred to the CS collection of Dr I. T. Riley. The isolate has been included in previously published studies [13–17, 32], and in these was grouped with other *C. toxicus* strains. More recent unpublished biochemical and molecular biological data also indicate, however, that the isolate in the CS collection may not be *C. toxicus*. It is possible that the culture was mislabelled, or became contaminated with a *C. rathayi* strain. The identity and taxonomic position of this strain needs to be resolved as it is one of only a few available *C. toxicus* reference strains from WA.

The WA *C. toxicus* field isolates fell into four ETs, and those in each ET may be considered as separate strains. Those forming the major strain, in ET 26, were found in the three distinct geographical locations in southwest WA over the 2-year sampling period. There also is evidence that this strain was present in WA prior to 1987, since the reference strain CS 28, sampled in that year, also was found in this ET. This strain has been shown to be sensitive to a specific bacteriophage believed to be associated with toxin production [16, 33]. Hence ET 26 may represent a major pathogenic strain of *C. toxicus* in WA, and its presence could have important implications for control strategies for ARGT in the state. The biological properties of other isolates in this ET, such as their toxin production, should be examined. It also would be useful to undertake MEE analysis on *C. toxicus* isolates from other sampling locations in WA, and to examine more isolates from SA, NSW and South Africa to confirm whether the species is made up of only a small number of closely-related clones.

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