Received: 2 November 2021 | Revised: 10 February 2022 | Accepted: 15 February 2022

DOI: 10.1111/jam.15500

Journal of Applied Microbiology

ORIGINAL ARTICLE

Esculin hydrolysis negative and TcdA-only producing strains of *Clostridium (Clostridioides) difficile* from the environment in Western Australia

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Funding information
Mahidol University; National Health and Medical Research Council, Grant/Award Number: APP1138257

Abstract

Background and Aims: *Clostridium (Clostridiodes) difficile* clade 3 ribotype (RT) 023 strains that fail to produce black colonies on bioMérieux ChromID agar have been reported, as well as variant strains of *C. difficile* that produce only toxin A. We have recently isolated strains of *C. difficile* from the environment in Western Australia (WA) with similar characteristics. The objective of this study was to characterize these strains. It was hypothesized that a putative *β*-glucosidase gene was lacking in these strains of *C. difficile*, including RT 023, leading to white colonies.

Methods and Results: A total of 17 environmental isolates of *C. difficile* from garden soil and compost, and gardening shoe soles in Perth, WA, failed to produce black colonies on ChromID agar. MALDI-TOF MS analysis confirmed these strains as *C. difficile*. Four strains contained only a *tcdA* gene ($A^+B^-CDT^-$) by PCR and were a novel RT (QX 597). All isolates were susceptible to all antimicrobials tested except one with low-level resistance to clindamycin (MIC = 8 mg/L). The four *tcdA*-positive strains were motile. All isolates contained neither *bgl* locus but only *bgl K* or a putative *β*-glucosidase gene by PCR. Whole-genome sequencing showed the 17 strains belonged to novel multi-locus sequence types 632, 848, 849, 850, 851, 852 and 853, part of the evolutionarily divergent clade C-III. Four isolates carried a full-length *tcdA* but not *tcdB* nor binary toxin genes.

Conclusions: ChromID *C. difficile* agar is used for the specific detection of *C. difficile* in the samples. To date, all strains except RT 023 strains from clinical samples hydrolyse esculin. This is the first report to provide insights into the identification of esculin hydrolysis negative and TcdA-only producing ($A^+B^-CDT^-$) strains of *C. difficile* from environmental samples.

Significance and Impact of the Study: White colonies of *C. difficile* from environmental samples could be overlooked when using ChromID *C. difficile* agar, leading to false-negative results, however, whether these strains are truly pathogenic remains to be proven.

KEYWORDS

clade-III, *Clostridium (Clostridioides) difficile*, environment, esculin hydrolysis negative, putative *β* glucosidase gene, toxin A variant, whole-genome sequencing

INTRODUCTION

Clostridium (*Clostridioides*) *difficile* is a Gram-positive, opportunistic, spore-forming, anaerobic bacterium, the etiological agent of antimicrobial-associated diarrhoea and pseudomembranous colitis in hospitalized patients (Smits et al., 2016). However, increasing rates of community-associated *C. difficile* infection (CA-CDI), accounting for up to 50% of all CDI cases in the United States (Guh et al., 2020) and 26% of cases in Australia (Slimings et al., 2014), suggest exposure to various sources of *C. difficile* external to the healthcare system such as soil, water and wastewater, seafood, animals, meat and meat products and vegetables (Bloomfield & Riley, 2016; Hensgens et al., 2012). There have been reports of overlapping ribotypes (RTs) of *C. difficile* found in animals and humans (Anderson et al., 2017; Jhung et al., 2008) suggestive of zoonotic transmission (Jhung et al., 2008; Rupnik, 2007). The application of compost and manure containing *C. difficile* to gardens has led to the dissemination of *C. difficile* in the domestic environment (Dharmasena & Jiang, 2018; Shivaperumal et al., 2020), and contamination of vegetables (Lim et al., 2018a; Metcalf et al., 2010). Also, shoe soles are likely to carry *C. difficile* from gardens of all types into the built environment including healthcare systems (Janezic et al., 2018; Shivaperumal et al., 2020).

Chromogenic agars have become popular bacteriological media for the isolation of a variety of pathogens from clinical and environmental sources, including *C. difficile*. *C. difficile* ChromID™ agar (bioMérieux, Marcy l'Etoile, France) gives black colonies of *C. difficile* in 24 h following hydrolysis of the substrate and a reaction with ferric citrate (Eckert et al., 2013). Production of distinct black colonies on ChromID agar improves the presumptive identification of *C. difficile* from complex samples (Connor et al., 2016). To date, at least one clinically relevant ribotype (RT) of *C. difficile*, RT 023, a binary toxin-positive (CDT+) strain, produces colourless colonies on ChromID agar after anaerobic incubation (Connor et al., 2016). It was hypothesized that *bglA* cluster 1 (encoding *β*-glucosidase) was associated with the white colony phenotype (Shaw et al., 2020) and that the phenotype may depend on the presence of another putative *β*-glucosidase gene in these strains of *C. difficile* (Connor et al., 2016).

In a recent study of home gardens in Perth, Western Australia (WA), 17 strains of *C. difficile* were recovered that failed to produce black colonies on *C. difficile* ChromID™ agar (Shivaperumal et al., 2020). The objective of the present study was to further characterize these strains phenotypically and genotypically.

MATERIALS AND METHODS

Strains

The 17 putative esculin hydrolysis-negative strains of *C. difficile*, all isolated in Perth, WA, together with comparator strains, are shown in Table 1.

Confirmation of strain identity

MALDI-TOF MS was carried out as described previously by Schulthess et al. (2013). Analysis of mass spectra was performed with a Microflex LT mass spectrometer (Bruker Daltonik) using the MALDI-bioTyper 3.0 software (Bruker Daltonics). The isolates were tested using *C. diff* Quik Chek Complete® (Techlab), a rapid membrane enzyme immunoassay (EIA) for the simultaneous detection of *C. difficile* glutamate dehydrogenase (GDH) and toxins A and B. A volume of 25 µl of a 48 h brain heart infusion broth culture (1 McFarland unit turbidity) and appropriate controls were used according to the manufacturers' instructions. All isolates were further confirmed using L-proline aminopeptidase DIATABS™ (Rosco Diagnostica) as described by the manufacturer.

Phenotypic characteristics

Motility

Motility assays were conducted according to the procedure described by (Hong et al., 2019). Briefly, isolates and esculin hydrolysis-positive (R20291) and -negative (R10725) controls were cultured on blood agar incubated anaerobically for 48 h. Brain heart infusion broth with 0.175% agar in test tubes was inoculated with a 1 µl loopful of colonies by stabbing the top 5 mm of sloppy agar with the loop. The tubes were incubated anaerobically at 37°C for 24 h and growth was monitored. Motility was described by the distance of movement of bacteria from the inoculation line in the low concentration agar and scored as nonmotile (0–2 mm), motile (2–5 mm) and highly motile (>5 mm).

TABLE 1 Summary of *C. difficile* and comparator strains analysed in this study

Strain	Ribotype	Toxin profile	Sequencetype	Reference	Source
HGP05	125	A-B-CDT-	848	Shivaperumal et al. (2020)	Soil
HGP07	UNIQUE	A-B-CDT-	849	Shivaperumal et al. (2020)	Soil
HGP14	QX597	A + B-CDT-	632	Shivaperumal et al. (2020)	Soil
HGP19	125	A-B-CDT-	848	Shivaperumal et al. (2020)	Soil
HGP28	QX639	A-B-CDT-	850	Shivaperumal et al. (2020)	Soil
HGP30	QX597	A + B-CDT-	632	Shivaperumal et al. (2020)	Soil
HGP33	QX597	A + B-CDT-	632	Shivaperumal et al. (2020)	Shoe sole
HGP34	QX638	A-B-CDT-	851	Shivaperumal et al. (2020)	Soil
HGP35	QX639	A-B-CDT-	850	Shivaperumal et al. (2020)	Soil
HGP43	125	A-B-CDT-	848	Shivaperumal et al. (2020)	Compost
HGP46	QX637	A-B-CDT-	852	Shivaperumal et al. (2020)	Soil
HGP47	UNIQUE	A-B-CDT-	853	Shivaperumal et al. (2020)	Soil
HGP48	QX637	A-B-CDT-	852	Shivaperumal et al. (2020)	Soil
HGP71	125	A-B-CDT-	848	Shivaperumal et al. (2020)	Compost
HGP78-1	QX597	A + B-CDT-	632	Shivaperumal et al. (2020)	Soil
HGP78-2	125	A-B-CDT-	848	Shivaperumal et al. (2020)	Soil
HGP79	125	A-B-CDT-	848	Shivaperumal et al. (2020)	Compost
R8375	002	$A^+B^+CDT^-$		N/A	ECDC
Clos di 21	023	$A^+B^+CDT^+$		N/A	ECDC
R11446	014	$A^+B^+CDT^-$		N/A	ECDC
2149/ATCC 43600	014	$A^+B^+CDT^-$		N/A	ECDC
1470 / ATCC 43598	017	$A^-B^+CDT^-$		N/A	ECDC
R10725	078	$A^+B^+CDT^+$		N/A	ECDC
R20291 / NCTC 13366	027	$A^+B^+CDT^+$		N/A	ECDC
VPI 10463 / ATCC 43255	087	$A^+B^+CDT^-$		N/A	ECDC
CD630 / ATCC BAA-1382™	012	$A^+B^+CDT^-$		N/A	ECDC
ES1213	251	$A^+B^+CDT^+$		Wehrhahn et al. (2019)	Human

In vitro antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of a panel of nine antimicrobial agents were determined using the agar dilution method as described by the Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2013; CLSI, 2019). Brucella agar supplemented with hemin [5 μg/ml], vitamin K_1 [1 μg/ml] and laked sheep blood [5%, vol/vol] was used with a slight modification of the CLSI guidelines (CLSI, 2007), and laked horse blood was used. The panel consisted of the first-line therapy drugs for CDI; vancomycin, metronidazole and fidaxomicin, together with clindamycin, erythromycin, moxifloxacin, amoxicillin-clavulanate, meropenem and rifampin. MIC breakpoints for vancomycin and metronidazole were based on the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://eucast.org). A MIC breakpoint of ≥1 mg/L for fidaxomicin was based on the European Medicines Agency (EMA) proposal (report WC500119707; http://www.ema.europa.eu/) while the breakpoints for clindamycin, erythromycin, amoxicillin-clavulanate, moxifloxacin and meropenem were those provided by the CLSI (CLSI, 2013). Breakpoints for rifampin have not been published by CLSI and susceptibility was categorized according to MICs (⩽0.002 mg/L, susceptible; 0.003–32 μg/ml, intermediate and >32 μg/ml, resistant) (Curry et al., 2009).

Growth kinetics assay

The toxin A (A + B-CDT-) positive isolates and comparator strains CD630/ATCC BAA-1382 (RT 012), a historic low toxin producer, ATCC 700057 (RT 038), a known

nontoxigenic strain, ATCC 43255 (RT 087), a known high toxin producer and Clos di 21 (RT 023), a known white colony producing strain were used as reference strains for growth kinetics assays. Preparation of a standard inoculum and the *C. difficile* growth kinetics assays were performed in triplicate according to methods described elsewhere (Hong et al., 2019).

Bgl gene PCR

C. difficile crude template DNA was prepared by resuspension of 48 h culture cells in a 5% Chelex-100® resin (Biorad) solution. PCR was used to characterize the esculin hydrolysis-negative *C. difficile* isolates by determining the presence of a *β*-glucosidase gene. Primers were designed for detecting the gene using the NCBI Primer-BLAST software programme https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi. The primer sequences were verified using http://insilico.ehu.es/PCR/index.php?mo=Clostridium; the novel primers were putative *bgl* F (forward: 5′-GGAGGTTGGTTAGTTTTAGA-3′ [20 bp]), putative *bgl* R (reverse: 5′-AGGATACCATTCTTGAGCT-3′ [19 bp]). Reaction mixes (total volume of 25 µl) consisted of 5 µl of template DNA, 4 mM $MgCl_2$, 400 µM dNTP, 0.4 µM of each primer, 3.75 U AmpliTaq Gold® DNA polymerase and 0.024% BSA. Reactions were run with an initial denaturation step of 94°C for 3 min, followed by 35 amplification cycles of 93°C for 15 s, 50°C for the 30 s and 68°C for 30 s with the final extension step of 68°C for 10 min. PCR product (size 1131 bp) was analysed on the QIAxcel Advanced capillary gel electrophoresis platform (QIAGEN, Venlo). Esculin hydrolysis-positive strains from clade 1 (RT 012 and RT 014), clade 2 (RT 027 and RT 251), clade 4 (RT 017) and clade 5 (RT 078) were used as positive controls while the RT023 esculin hydrolysis-negative strain from clade 3 and ultra-pure H_2O were the negative controls.

Genome analysis

Whole-genome sequencing (WGS) was performed on all 17 esculin hydrolysis-negative strains of *C. difficile*. Genomic DNA extraction, library preparation, WGS (Illumina NovaSeq), and assembly and annotation of draft genomes were performed as previously described (Knight et al., 2019). Evolutionary relatedness between the esculin hydrolysis-negative strains was determined by multi-locus sequence typing (PubMLST, https://pubmlst.org/cdifficile/), and core genome single-nucleotide polymorphism (cgSNP) analysis (Knight et al., 2019).

A neighbour-joining tree of concatenated MLST alleles was generated in MEGA v10 and annotated using iToL v4 [https://itol.embl.de/]. ResFinder, Phaster and Artemis were used to identify and characterize accessory AMR loci, phages and toxin genes respectively. WGS data have been submitted to the NCBI Short Read Archive under Bioproject PRJNA772357 (accessions SAMN22374628-SAMN22374644).

The complete PaLoc sequence was extracted from the genome of *C. difficile* strains 630 [GenBank Accession Number AM180355.1], RA09-70 [GenBank Accession Number JPPA00000000, (Monot et al., 2015)] and the 17 esculin hydrolysis-negative strains and annotated using Prokka v1.13.3 (https://github.com/tseemann/prokka). The extracted segments were compared and visualized using Clinker v0.0.12 (https://github.com/gamcil/clinker).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 software [GraphPad Software]. One-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test was used to assess the significance in growth rates in each strain and the comparator strains.

RESULTS

Phenotypic characterization

All 17 putative esculin hydrolysis-negative strains failed to produce distinctive black colonies characteristic of *C. difficile* on bioMérieux ChromID plates after 24 or 48 h anaerobic incubation. The colonies were white and umbonate, with irregular edges, and otherwise resembled those of *C. difficile* RT 023 (Figure 1**)**. Using a MALDI Bio-Typer, all strains were identified as *C. difficile*, and all were positive for the detection of L-proline aminopeptidase.

Glutamate dehydrogenase, toxin production, toxin gene PCR and ribotyping

All 17 isolates produced GDH, four (24%) were toxigenic and, by PCR ribotyping, had similar banding patterns; these were given an internal RT of QX 597. Six isolates were nontoxigenic RT 125. The remaining seven isolates

were also nontoxigenic: five were also given a QX prefix, QX 637 (two strains), QX 638 and QX 639 (two strains), and two were unique singletons having never been seen before in our laboratory. No binary toxin genes were present (Table 1).

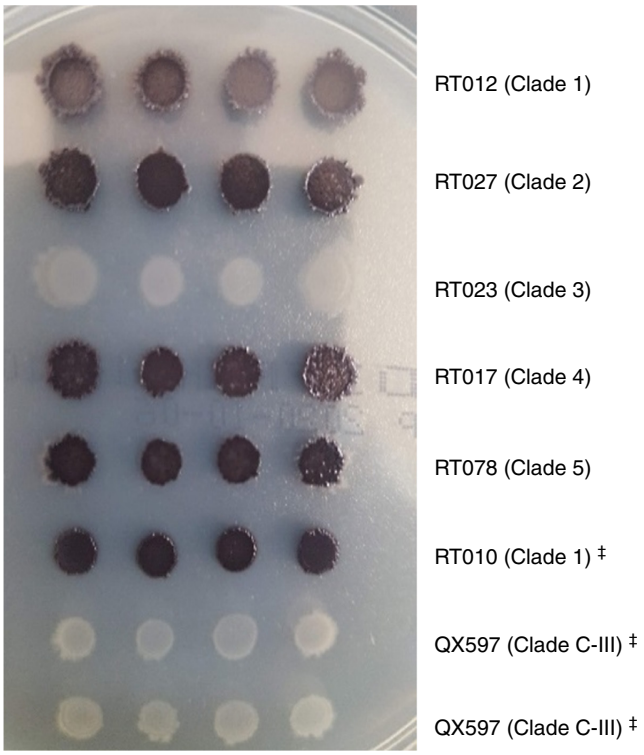


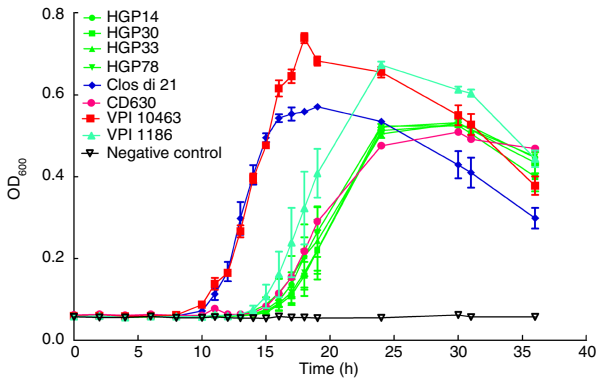
FIGURE 1 *C. difficile* colonies on *C. difficile* ChromID agar. Standard black colonies produced by esculin hydrolysis-positive strains of *C. difficile* clade 1 (RT012), clade 2 (RT027), clade 4 (RT017), clade 5 (RT078) and garden strain‡ RT010, on ChromID. Colourless colonies produced by esculin hydrolysis-negative strains of *C. difficile* clade 3 (RT023) and garden strain QX597

Growth kinetics

Growth kinetics assays for esculin hydrolysis-positive and -negative strains of *C. difficile* were performed to define any differences in growth characteristics (Figure 2). While there was no growth in the first 6 h, there were obvious differences during log and stationary phases between different strains. The cell density increased from 9 h for the control strains VPI10463 and Clos di 21/RT 023, and at 15 h in the esculin-negative variant strains. At 18 h, VPI10463 attained its maximum growth compared to Clos di 21, VPI11186 and CD630. However, the duration of the log phase in the control strains was similar except for VPI11186. The esculin hydrolysis-negative strains had a prolonged exponential phase (13 h) and diminished growth was observed throughout the 36 h period compared to control strains tested. The highest cell density of the esculin-negative isolates was only one-third of the control strain, VPI10463. Thus, while the overall growth rate of esculin-negative strains was not significantly different to CD630 and VPI11186, it was when compared with the control strains Clos di 21 ($p < 0.05$) and VPI10463 ($p < 0.001$).

Motility

Motility was examined by stab inoculation into standard motility agar (0.175%) assessing swimming motility after 24 h incubation. Ten isolates including four toxin A-positive strains displayed a high diffusion of growth away from the stab inoculum suggesting a high motile phenotype (mean projection length = 7 mm), whereas seven isolates appeared as average motility with a projection length of 3 mm and two isolates were nonmotile (mean projection length = 1.5 mm). *C. difficile* strains R20291 (RT 027) and



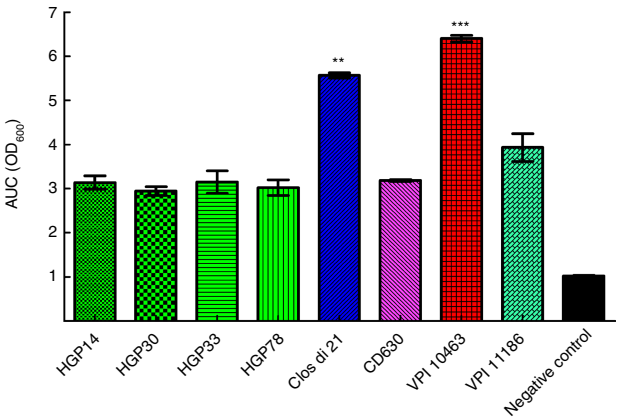


FIGURE 2 Growth kinetics of esculin hydrolysis-negative and reference strains. The growth of strain clos di 21 (RT023), VPI10463 (RT087), CD630 (RT012), VPI11186 (RT038) and esculin hydrolysis strains was measured by OD_{600} up to 36 h

R10725 (RT 078) were used as positive and negative controls respectively. While the positive control R20291 was highly motile (mean projection length = 8 mm), negative control, R10725 was nonmotile, with an average projection length of 1.5 mm. All the isolates were further confirmed by a hanging drop motility test. The flagellin *fliC* and flagellar cap *fliD* genes were conserved in all strains including in toxin A-positive strains. All isolates contained F1 (late-stage flagella genes), F3 (early-stage flagella genes) and four additional late-stage flagella genes including in reference strains (RT 027, RT 023 and RT 012).

Antimicrobial susceptibility

All isolates were susceptible to metronidazole, fidaxomicin, erythromycin, moxifloxacin, amoxicillin-clavulanate and rifampin. Fidaxomicin (MIC_{50}/MIC_{90}/GMs, 0.12/0.25/0.16 mg/L) and metronidazole (MIC_{50}/MIC_{90}/GMs, 0.5/0.5/0.38 mg/L) were the most potent agents and a range of MICs was seen (fidaxomicin: MIC_{range} = 0.25–0.5; metronidazole: MIC_{range} = 0.06–0.25. Although elevated MICs were not observed for clindamycin for most isolates (65%, 11/17), five (29%) showed intermediate susceptibility (MIC = 4 mg/L) and one (6%) showed resistance (MIC = 8 mg/L). While three isolates were resistant (MIC = 8 mg/L) to meropenem (MIC_{50}/MIC_{90}/GMs, 4/8/4.52 mg/L), almost 75% of the toxigenic strains were resistant to clindamycin (MIC_{50}/MIC_{90}/GMs, 2/4/1.84 mg/L) (Table 2).

Genome analysis

WGS was successfully performed on the 17 environmental strains. Genome metrics and general genomic features evaluated are shown in Table 3. Variations in genome size and content were found in all 17 genomes with sizes ranging from ~4.1 to ~4.5 Mb (median 4.18) and the GC percentage ranging between 28.23 and 28.56% (median 28.41). An MLST phylogeny for the 17 esculin hydrolysis-negative strains is shown in Table 4. The 17 genomes sequenced belonged to one of seven novel STs within evolutionarily divergent clade C-III: STs 632, 848, 849, 850, 851, 852 and 853 (Figure 3). All *tcdA*-positive isolates (novel RT QX597, n = 4) belonged to ST632. The predominant RT among the 17 strains (RT 125, $n = 6$) belonged to ST848 and the unique RTs belonged to STs 853 ($n = 1$) and 849 ($n = 1$). The remaining five isolates belonged to three unique STs (STs 850, $n = 2$, 851, $n = 1$ and 852, $n = 2$). All seven STs belonged to evolutionary clade C-III (Table 4).

A few strains that shared postcodes and a temporal relationship were located in distant parts of phylogeny with different STs suggesting genetic heterogeneity, for example, postcode 6024—ST848 (different soil samples and compost, RT 125), ST632 (soil, RT QX597), postcode 6152—ST852 (soil, RT QX637) and postcode 6152—ST853 (soil, unique RT), postcode 6009—ST850 (soil, RT QX639) and postcode 6009—ST851 (soil, RT QX638). However, some strains showed clustering in distant postcodes without a temporal relationship, for example, postcodes 6152 (ST853, soil, unique RT), 6059 (ST849, soil, unique RT), 6163 (ST848, soil, RT 125), 6014 (ST848, compost, RT 125), 6151 (ST848, compost, RT 125), 6024 (ST848, soil and compost, RT 125). Similarly, toxin A-positive isolates (soils and shoe sole sample, postcodes 6024, 6054 and 6006, ST632, RT QX597) were notably clustering with nontoxigenic strains (soils, postcode 6152, RT QX637). This information suggests these strains shared a recent ancestry and possible long-range transmission.

Confirming the PCR result of four strains positive for one of the major virulence factors (*tcdA*), in silico analysis confirmed that all toxigenic strain genomes were positive for full-length *tcdA* but negative for *tcdB* and *cdtA/B*. Also, syntenic holin genes *uviA* and *uviB* were found next to *tcdA* in all four toxin A PaLocs (Figure 4). A complete PaLoc was absent in the remaining 13 nontoxigenic strains. Among the 17 strains, seven STs demonstrated allelic conservations in some housekeeping genes and some differed by polymorphisms.

In concordance with the phenotypic testing, in silico antimicrobial resistance (AMR) profiling demonstrated that all 17 sequenced genomes did not contain accessory genes conferring AMR.

Genomes of esculin hydrolysis-negative strains were screened by PCR for a β-glucosidase gene encoding hydrolase activity. In the black colonies producing strains from clades 1, 2, 4 and 5, a 1131 bp putative β glucosidase gene fragment was found whereas clade 3 RT 023 and all the esculin hydrolysis-negative isolates did not contain such a fragment.

A threshold of 0–2 cgSNPs was used to determine clonal relatedness among the isolates (Eyre et al., 2017; Knight et al., 2017). A heatmap of pairwise cgSNPs differences between all 17 genomes is shown in Table 5. Overall, 11.8% (2/17) of strains showed a clonal relationship between the strains and 75% ($n = 3$) of compost samples showed a clonal relationship (2 cgSNPs, ST848) with other compost samples ($n = 4$) from two suburbs (~12 km distance). Other strains showed a relatively close relationship among soil ($n = 2$) and composts ($n = 4$) samples from three suburbs (~3–10 cgSNPs, an average of 15 cgSNPs between strains with an average distance of ~43 km away) and compost ($n = 4$) and soil ($n = 3$) from six suburbs (~11–20 cgSNPs, an average of 13.5 cgSNPs, ~65 km) in WA. All these strains

TABLE 2 Antimicrobial susceptibility analysis

	Antimicrobial breakpoint (mg/L)[a]																										
	FDX			VAN			MTZ			RIF			CLI			ERY			AMC			MXF			MEM		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Strain	-	-	≥1	≤2	-	>2	≤2	-	>2			≥32	≤2	4	≥8	-	-	>8	≤4	8	≥16	≤2	4	≥8	≤4	8	≥16
HGP05	0.12			2			0.25			2			4			0.5			0.25			1			4		
HGP07	0.25			2			0.5			2			2			0.5			0.5			1			4		
HGP14	0.12			2			0.25			4			2			1			0.25			2			4		
HGP19	0.25			2			0.5			1			0.5			0.5			0.5			1			4		
HGP28	0.25			2			0.5			4			0.5			1			0.5			2			8		
HGP30	0.25			2			0.25			4			4			1			0.25			1			4		
HGP33	0.25			2			0.25			4			4			1			0.25			2			4		
HGP34	0.12			2			0.5			4			2			0.5			0.5			1			4		
HGP35	0.12			2			0.5			2			0.5			1			0.5			2			8		
HGP43	0.12			2			0.5			4			2			0.5			0.5			1			4		
HGP46	0.12			1			0.5			8			2			1			0.5			1			8		
HGP47	0.12			2			0.25			1			4			1			0.5			1			4		
HGP48	0.25			1			0.5			8			2			1			0.5			1			4		
HGP71	0.12			2			0.5			4			0.5			0.25			0.5			1			4		
HGP78-1	0.25			2			0.25			4			8			1			0.25			1			4		
HGP78-2	0.25			2			0.5			4			4			0.5			0.5			1			4		
HGP79	0.06			2			0.25			0.5			1			0.25			0.5			1			4		
C. difficile 700,057	0.015			2			0.125			0.004			8			1			0.5			2			2		
B. fragilis 25,285	>4			>4			0.5			0.25			1			16			0.25			0.25			<0.25		
E. lentum 43,055	0.03			2			0.25			0.06			0.125			0.25			0.5			0.25			0.5		
B. thetaiotamicron 29,741	>4			>4			>4			1			2			16			0.25			1			<0.25		

Note: S, susceptible; I, intermediate; R, resistant. ATCC700057 (*C. difficile*) and ATCC25285 (*Bacteroides fragilis*) strains were controls as recommended by CLSI. FDX, fidaxomicin; VAN, vancomycin; MTZ, metronidazole; RIF, rifampin; CLI, clindamycin; ERY, erythromycin; AMC, amoxicillin-clavulanate; MXF, moxifloxacin; MEM, meropenem.

[a]Breakpoints for fidaxomicin recommended by EMA (report WC500119707, http://www.ema.europe.eu/) (European Medicines Agency, 2011), for vancomycin and metronidazole recommended by EUCAST (EUCAST, 2020), remaining as recommended by CLSI (CLSI, 2013).

TABLE 3 Genome metrics

Strain	ENA accession	N contigs	Total length (bp)	N50 (bp)	GC (%)	Median seq depth
HGP05	SAMN22374628	13	4,138,029	3,844,413	28.48	55.26
HGP07	SAMN22374629	14	4,119,880	3,757,367	28.51	77.33
HGP14	SAMN22374630	81	4,241,508	93,313	28.45	99.15
HGP19	SAMN22374631	14	4,136,262	3,782,574	28.45	45.83
HGP28	SAMN22374632	12	4,243,128	3,951,787	28.38	36.77
HGP30	SAMN22374633	92	4,265,720	92,878	28.42	101.48
HGP33	SAMN22374634	13	4,249,315	3,787,856	28.48	80.19
HGP34	SAMN22374635	11	4,237,441	2,696,177	28.44	68.39
HGP35	SAMN22374636	16	4,241,733	3,836,539	28.38	33.77
HGP43	SAMN22374637	13	4,137,439	3,843,029	28.47	93.05
HGP46	SAMN22374638	9	4,192,908	3,967,814	28.23	48.57
HGP47	SAMN22374639	12	4,511,861	4,285,437	28.33	29.15
HGP48	SAMN22374640	10	4,206,389	3,980,098	28.28	107.63
HGP71	SAMN22374641	13	4,146,017	3,852,144	28.47	60.05
HGP78-1	SAMN22374642	77	4,485,801	104,641	28.24	95.72
HGP78-2	SAMN22374643	13	4,171,210	3,877,171	28.41	28.68
HGP79	SAMN22374644	13	4,181,177	3,842,470	28.42	38.25

TABLE 4 MLST data

Strain	ST	Clade	*Adk*	*atpA*	*Dxr*	*glyA*	*recA*	*sodA*	*Tpi*
HGP05	848	C-III	86	89	86	125	73	100	106
HGP07	849	C-III	87	89	86	125	73	101	106
HGP14	632	C-III	57	61	61	90	50	71	83
HGP19	848	C-III	86	89	86	125	73	100	106
HGP28	850	C-III	88	39	54	57	74	50	56
HGP30	632	C-III	57	61	61	90	50	71	83
HGP33	632	C-III	57	61	61	90	50	71	83
HGP34	851	C-III	89	90	87	126	75	102	107
HGP35	850	C-III	88	39	54	57	74	50	56
HGP43	848	C-III	86	89	86	125	73	100	106
HGP46	852	C-III	23	91	88	127	76	37	108
HGP47	853	C-III	87	89	86	128	77	103	106
HGP48	852	C-III	23	91	88	127	76	37	108
HGP71	848	C-III	86	89	86	125	73	100	106
HGP78-1	632	C-III	57	61	61	90	50	71	83
HGP78-2	848	C-III	86	89	86	125	73	100	106
HGP79	848	C-III	86	89	86	125	73	100	106

Source: 1 (soil); 2 (shoes); 3 (compost), ST: Sequence type, RT: Ribotype, TP: Toxin profile.

belonged to RT 125 and the new ST (848). However, all the toxigenic isolates ($n = 5$) from three suburbs (~10–32 cgSNPs, an average of 19.1 cgSNPs, ~28 km) belonged to a novel RT (597) and a new ST (632). All these strains shared recent evolutionary history.

DISCUSSION

Recently, there has been an increased incidence of CA-CDI, now comprising ~50% of all CDI cases in the USA (Guh et al., 2020) and suggesting that sources of *C. difficile*

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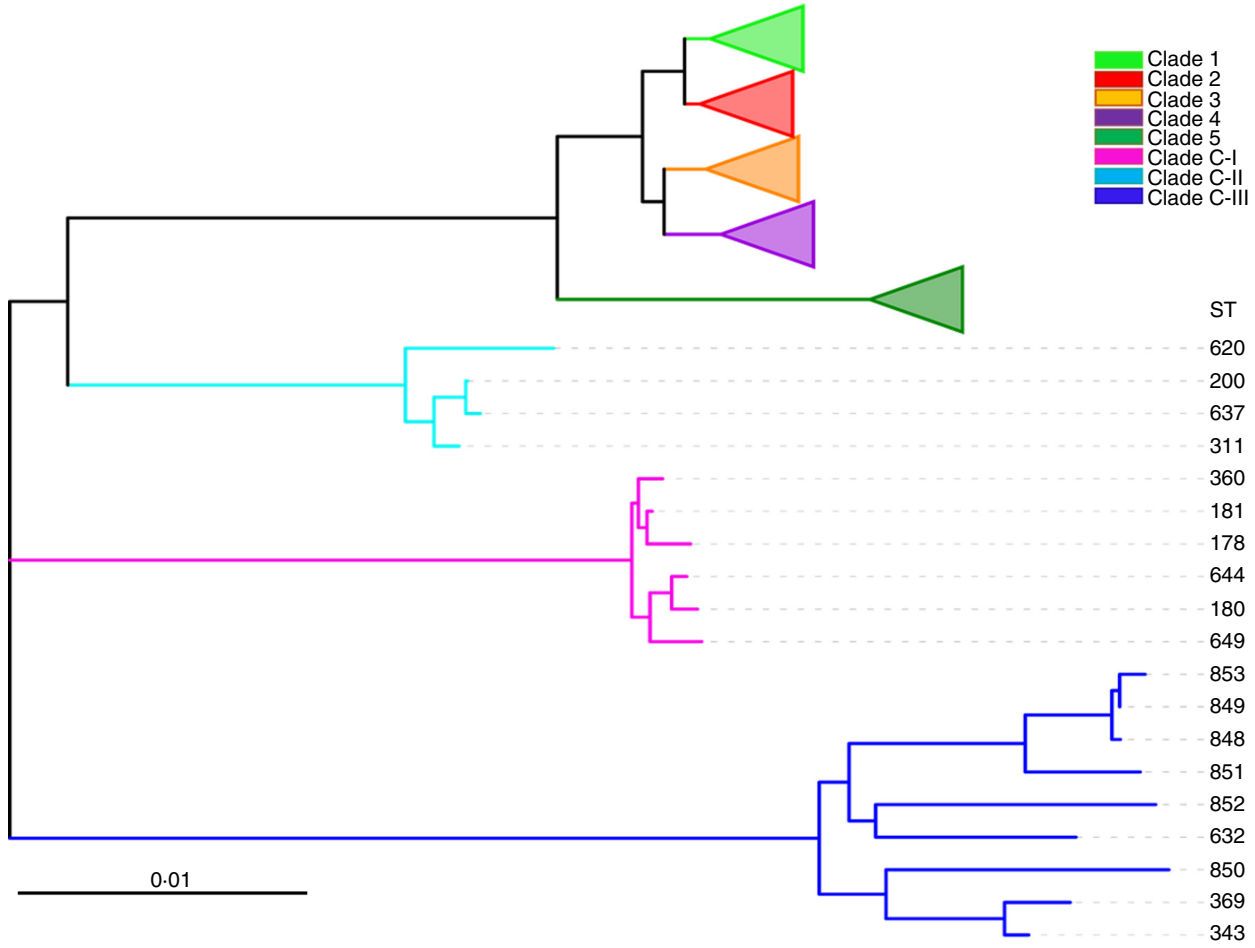

FIGURE 3 Global phylogenetic context of seven novel sequence types. MLST phylogeny based on concatenated allele sequences for seven novel sequence types (cryptic clade C-III) and well-characterized representatives of MLST clade 1 (ST54, RT012), clade 2 (ST1, RT027), clade 3 (ST22, RT023), clade 4 (ST37, RT017), clade 5 (ST11, RT078), as well as other cryptic clades C-I (ST360) and C-II (ST200). The scale shows the number of substitutions per site

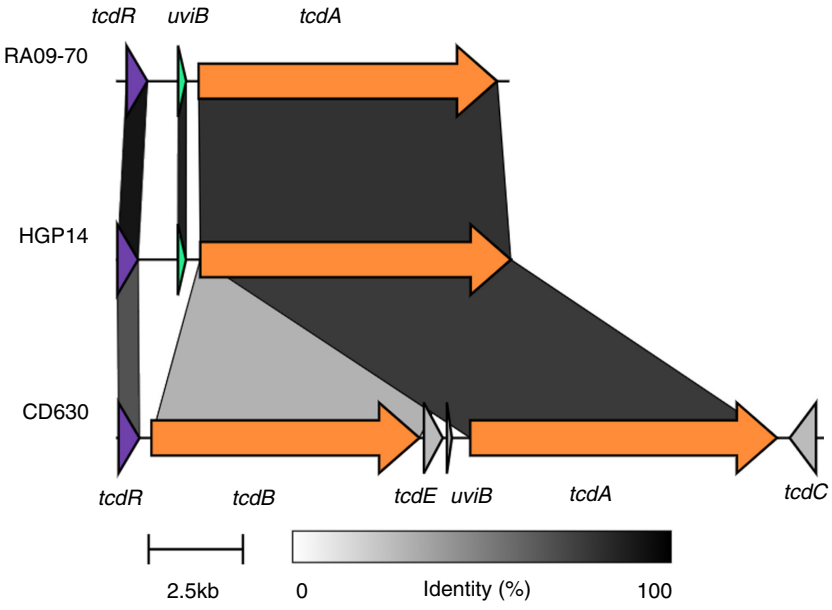

FIGURE 4 PaLoc structure of *tcdA*-positive isolates of *C. difficile* from environmental samples. Mono-toxin PaLoc of environmental isolates (e.g. HGP14) shared synteny and a high nucleotide sequence similarity to the PaLoc of clinical isolate RA09-70 (clade 5)

TABLE 5 Core genome SNP analysis

	HGP46	HGP48	HGP28	HGP35	HGP14	HGP30	HGP33	HGP78-1	HGP34	HGP47	HGP07	HGP05	HGP19	HGP78-2	HGP43	HGP71	HGP79
ST[a]	852	852	850	850	632	632	632	632	851	853	849	848	848	848	848	848	848
RT[b]	QX637	QX637	QX639	QX639	QX597	QX597	QX597	QX597	QX638	UNIQUE	UNIQUE	125	125	125	125	125	125
TP[c]	A-B-CDT-	A-B-CDT-	A-B-CDT-	A-B-CDT-	A+B-CDT-	A+B-CDT-	A+B-CDT-	A+B-CDT-	A-B-CDT-	A-B-CDT-	A-B-CDT-	A-B-CDT-	A-B-CDT-	A-B-CDT-	A-B-CDT-	A-B-CDT-	A-B-CDT-
Source[d]	1	1	1	1	1	1	2	1	1	1	1	1	1	1	3	3	3
Post code	6152	6152	6010	6009	6006	6054	6054	6024	6009	6152	6059	6059	6163	6024	6014	6151	6024
HGP46		16	6207	6207	5128	5118	5122	5122	6203	6133	6150	6092	6098	6097	6096	6096	6095
HGP48	16		6203	6203	5124	5114	5116	5118	6199	6133	6148	6092	6096	6095	6094	6094	6093
HGP28	6207	6203		28	5112	5104	5106	5106	5125	5076	5079	5037	5037	5042	5041	5039	5042
HGP35	6207	6203	28		5116	5106	5108	5108	5129	5082	5083	5041	5041	5046	5045	5043	5046
HGP14	5128	5124	5112	5116		18	16	20	5103	5008	5029	4975	4985	4984	4983	4983	4982
HGP30	5118	5114	5104	5106	18		10	10	5101	5006	5027	4975	4983	4982	4981	4981	4980
HGP33	5122	5116	5106	5108	16	10		14	5099	5008	5027	4975	4983	4982	4981	4981	4980
HGP78-1	5122	5118	5106	5108	20	10	14		5101	5008	5029	4977	4985	4984	4983	4983	4982
HGP34	6203	6199	5125	5129	5103	5101	5099	5101		2040	2026	1990	1985	1985	1985	1983	1986
HGP47	6133	6133	5076	5082	5008	5006	5008	5008	2040		572	578	579	581	577	577	580
HGP07	6150	6148	5079	5083	5029	5027	5027	5029	2026	572		535	532	534	528	528	529
HGP05	6092	6092	5037	5041	4975	4975	4975	4977	1990	578	535		17	17	11	11	14
HGP19	6098	6096	5037	5041	4985	4983	4983	4985	1985	579	532	17		14	8	6	11
HGP78-2	6097	6095	5042	5046	4984	4982	4982	4984	1985	581	534	17	14		10	8	11
HGP43	6096	6094	5041	5045	4983	4981	4981	4983	1985	577	528	11	8	10		2	3
HGP71	6096	6094	5039	5043	4983	4981	4981	4983	1983	577	528	11	6	8	2		5
HGP79	6095	6093	5042	5046	4982	4980	4980	4982	1986	580	529	14	11	11	3	5	

Heatmap of pairwise core genome SNP differences between 17 isolates: 0-20, 21-100, 101-3000, 3001-6000 and 6001-7000.

[a]ST: Sequence type, bRT: Ribotype, cTP: Toxin profile, dSource: 1 (soil); 2 (shoes); 3 (compost).

[Correction added on 20th April 2022, after first online publication: Table 5 footnotes have been included]

causing disease can be diverse (Eyre et al., 2013). In addition, *C. difficile* is now a ubiquitous environmental organism (Moono et al., 2017; Rodriguez et al., 2018). Produced by bioMérieux, ChromID *C. difficile* agar has become popular for rapidly putatively identifying *C. difficile* from both clinical and environmental samples (Eckert et al., 2013; Perry et al., 2010). *C. difficile* RT 023 is a clinically important strain of *C. difficile* that fails to produce distinctive black colonies on ChromID *C. difficile* agar plates (Connor et al., 2016). Recently, we reported similar strains of *C. difficile* producing atypical white colonies on ChromID *C. difficile* agar isolated from environmental samples. Of 87 strains of *C. difficile* recovered in that study, 17 (19.5%) were esculin hydrolysis-negative and produced white colonies (Shivaperumal et al., 2020).

Hydrolysis of esculin by a *β*-glucosidase produces D-glucose and esculetin (6,7-dihydroxycoumarin) which react with ferric ions to produce black colonies of *C. difficile* (Connor et al., 2016; Perry et al., 2007) and esculin hydrolysis with ferric chloride provides a zymogram technique to locate an appropriate *β*-glucosidase, esculinase (Kwon et al., 1994). The main disadvantage of using some substrates in media is that the complex formed by hydrolysis diffuses rapidly throughout the agar medium. In contrast, the derivative of esculin, 3, 4-cyclohexenoes culetin-7-*β*-D-glucoside (CHE- β-D-glucoside), remains highly insoluble and nondiffusible in the medium (James et al., 1996).

In *Escherichia coli*, several catabolic systems have been explored in the utilization of aromatic glucosides such as arbutin (Arb), salicin (Sal) and esculin (Esc) that encode a sugar-specific permease that is a part of the phosphotransferase system (PTS) and a phospho-glucosidase necessary for the hydrolysis of the sugar (Zangoui et al., 2015). Upon mutational activation, the silent operon becomes functional. For example the β-glucoside catabolic system involved in esculin utilization is the *bgl* operon and, once activated, either BglF transports esculin inside the cell or phospho β-glucosidase B encoded by *bglB* cleaves the phosphorylated esculin (Zangoui et al., 2015). A phospho-β-glucosidase gene, *bglA* in *Streptococcus mutans* UA159 genome, upon targeted inactivation of the gene, revealed the involvement of *bglA* in the hydrolysis of esculin (Old et al., 2006). A regulon from *S. mutans* encodes a β-glucoside-specific Enzyme II (EII) component (*bglP*) and *bglA*, and a chromosomal deletion in both genes resulted in its inability to hydrolyse esculin in the presence of glucose but retained its ability to breakdown esculin with the removal of glucose in the medium (Cote et al., 2000). In *E. coli*, the acquisition of a four-nucleotide insertion in the *bglA* gene leads to esculin hydrolysis resulting in enhanced steady-state levels of the *bglA* transcript (Zangoui et al., 2015). In this study, we hypothesized that the esculin hydrolysis-negative strains do not possess the locus for esculin metabolism including the *licT* gene, encoding for the transcriptional regulator, *bglF*, *bglA* and the putative *β*-glucosidase gene.

In *C. difficile*, a putative *β*-glucosidase gene has been found in clades 1, 2, 4 and 5 but not in clade 3. Shaw et al. (2020) identified five *bgl* clusters and putative esculinase clusters in the *C. difficile* strain 630 (clade 1, RT 012) genome include *bgl* genes *bglA*, *bglG* and *bglF* (Shaw et al., 2020). In our study, the putative *β*-glucosidase gene was absent in clade 3 and esculin hydrolysis-negative strains from environmental samples. The absence of *bglA* and *bglF* leads to an inability to hydrolyse esculin, however, *C. difficile* still utilizes carbohydrates from other sources.

The toxin bands in the *C. diff* Quik Chek Complete assay were weak for four strains with one showing

moderate intensity which has been described for a toxin A only positive (A + B-CDT-) clinical strain of *C. difficile* (Monot et al., 2015). MALDI-TOF MS was used also to confirm all isolates as *C. difficile*. Recent advances such as the availability of an extensive database and validated MALDI-TOF MS procedures showed the identification of anaerobes at species level has become more accurate (Kim et al., 2016). Despite these improvements in identifying bacterial pathogens at the species level, MALDI-TOF MS has not been used frequently for *C. difficile*. However, whether these strains are truly *C. difficile* is debatable given our recently published taxonomic study that suggests the cryptic clades are likely new species (Knight et al., 2021).

PCR ribotyping of all esculin-negative isolates demonstrated seven distinct RTs, including approximately one-third RT 125 (35.3%, 6/17) together with several novel RTs (Shivaperumal et al., 2020). In this study, in particular, we describe and characterize four isolates of toxigenic A + B-CDT- *C. difficile* from environmental samples. Monot et al. (2015) found the PaLoc of a toxin A-positive clinical strain contained *tcdR* and *tcdA*, but no *tcdB*, *tcdE* or *tcdC* genes (Monot et al., 2015). Instead, a new putative Coding DNA Sequence (CDS) of 216 bp, a substitution of *tcdE*, was found between *tcdR* and *tcdA* which encodes a domain with 71 amino acids homologous to the bacteriophage holin protein BhlA/UviB. They proposed a classical 'Bi-Toxin PaLoc' from the fusion of two 'Mono-Toxin PaLoc' from ancestral clostridia (Monot et al., 2015).

In a hamster model, a TcdA-only producing strain caused no mortality nor characteristic CDI signs including diarrhoea even though the strain was isolated originally from a patient with diarrhoea (Marvaud et al., 2019). Histology revealed that toxin A, presumably, caused significant caecal damage, inflammation with neutrophilic infiltration and extensive hyperplasia. It was thought that damage and inflammation were due to TcdA and flagellin as the TcdA only positive strain had regulons coding the flagellar proteins (Marvaud et al., 2019). In the current study, the flagellar genes *fliD* and *fliC* were present in all the TcdA (A + B-CDT-) isolates and these proteins are essential for functional flagella and bacterial adhesion to host cells (Stabler et al., 2009; Stevenson et al., 2015). An isogenic mutant producing only TcdA (A + B-CDT-) also caused disease and mortality in hamsters, however, this was delayed compared to A-B+ and wild-type strains (Kuehne et al., 2010). Thus, TcdA only producing strains of *C. difficile* causing human infection should not be ignored.

Fluoroquinolones, clindamycin and cephalosporins have been associated with a high risk of CDI (Kuijper et al., 2008). Previous studies showed high rates of resistance to clindamycin (69%) in *C. difficile* from pig samples even though clindamycin is not approved for use in food animals (Knight et al., 2017) and from compost and lawn samples (Lim et al., 2018b). In contrast, many esculin hydrolysis-negative strains from the present study were susceptible to clindamycin (68%), with some showing intermediate resistance (26%), in agreement with an earlier study from Europe (Janezic et al., 2012). In general, clindamycin-resistant strains show resistance to erythromycin owing to erythromycin ribosomal methylase gene class B (*ermB*) mediated resistance with the gene located on mobile genetic elements (Knight et al., 2017; Spigaglia, 2016). Notably, all isolates were susceptible to first-line CDI treatment drugs including metronidazole, vancomycin and fidaxomicin, similar to our earlier study (Knight et al., 2017).

Bacterial flagella contribute to the pathogenesis of disease through adherence, colonization and facilitating translocation of virulence factors which play a pivotal role in adapting to their biological niches. Flagellar motility is vital for the gastrointestinal bacteria including *C. difficile* to colonize in their hosts (Stevenson et al., 2015). The adherence of peritrichous flagella of *C. difficile* in mouse cecum revealed a 10-fold increase than the adherence of nonflagellated strains (Twine et al., 2009). In the present study, 26% of esculin hydrolysis strains demonstrated a high level of motility compared to the well-established epidemic strain RT 027 strain R20291. A similar result was observed in esculin hydrolysis-negative *C. difficile* RT 023 isolates in a recent study by Karpiński et al. (2022). All toxin A producing isolates were highly motile (>5 mm). All had the F1 (late-stage flagella genes) and four additional genes, DTDP-4-dehydrorhamnose reductase, glucose-1-phosphate thymidylyltransferase, DTDP-4-dehydrorhamnose 3,5-epimerase and DTDP-glucose 4,6-dehydratase, which are believed to play a role in virulence (Stabler et al., 2009). The F1 region of the genome encodes the genes for the structural proteins *fliD* and *fliC* essentially required for the formation of fully functional flagella (Stevenson et al., 2015). The *fliA* gene positively regulates the genes in the F1 region (El Meouche et al., 2013). However, other studies suggest that nonflagellated strains cause disease similar to flagellated strains (Stevenson et al., 2015) and the virulence is either RT dependent or nontraditional (Androga et al., 2019; El Meouche et al., 2013; Stabler et al., 2009).

Using WGS and high-resolution core genome phylogenetics and cgSNPs analysis we describe the evolutionary relationships of novel esculin hydrolysis-negative toxin A-positive strains. In silico MLST differentiated the 17 esculin hydrolysis-negative strains into seven novel STs (632, 848, 849, 850, 851, 852 and 853), all part of the evolutionarily divergent clade C-III. All *tcdA*-positive isolates belonged to sequence type 632. All toxigenic strains contained a full-length *tcdA*, but were negative for *tcdB* and

cdtA/B, and the PaLoc was located in different regions of the *C. difficile* genome. The insertion in the element of the *tcdA*-positive strains from this study was similar to that of the RA09-70 strain (Monot et al., 2015) (Figure 4).

Phylogenetic analysis of core genes can reveal ultrafine resolution of *C. difficile* populations (Dingle et al., 2011; Knight et al., 2017). In this study, there was diversity among isolates by RT and suburb, for example, RTs QX 597 (ST632) and RT 125 (ST848) were distributed in many suburbs. In some instances, soil and compost isolates collected from houses in different suburbs were closely related (2 cgSNPs) in their core genome suggesting long-range transmission. Overall, 75% of compost strains showed a clonal relationship with compost isolates from two suburbs approximately 12 km apart indicating possible contamination with animal manure of vegetables as the participants made their compost from vegetable waste at home. There was a high prevalence (56%) of *C. difficile* on root vegetables reported recently in WA which possibly contributes to contamination of traditional composts (Lim et al., 2018a).

Bacterial growth patterns obtained for various *C. difficile* isolates were similar to those seen in a previous study (Hong et al., 2019). However, the control strain VPI10463 reached exponential phase 2 h later than in the previous report (Hong et al., 2019), although the peak growth rate and the growth pattern were comparable. While the duration of the stationary phase was short in a high toxin producer (VPI10463, 6 h) (Hong et al., 2019), surprisingly, the white colony-producing strains had a prolonged stationary phase (16 h). The growth of all reference toxigenic strains was significantly better than that of CD630 ($p < 0.001$) (Vohra & Poxton, 2011). In this study, the toxin A producing white colony strains were significantly different from the control strains, Cl di 21 ($p < 0.05$) and VPI10463 ($p < 0.001$).

Our study had some limitations. First, the esculin hydrolysis-negative strains were found among 87 garden isolates recovered in the suburbs of Perth, Australia. Our results may not be reflective of the prevalence of esculin hydrolysis-negative strains in the rest of Australia due to the limited numbers of samples tested, and the isolation of Perth. Second, PCR for the putative *β*-glucosidase gene was performed on selected RTs from each clade and a wider range of RTs should be tested. Finally, until recently, toxin A is often produced with toxin B in both clinical and environmental strains. In this study, variant strains produced only toxin A and these strains should be tested in laboratory animals to demonstrate their pathogenicity and clinical features of any disease they cause.

In conclusion, we describe the isolation and characterization of esculin hydrolysis-negative *tcdA* only positive strains of *C. difficile* from the environment in WA. The white colonies seen on ChromID plates may affect the isolation and identification of toxin A only positive strains as they may be overlooked. A putative β-glucosidase gene was present in all clades except clade 3 and esculin hydrolysis-negative strains of *C. difficile*. The majority of *tcdA*-positive isolates were motile and belonged to novel RTs. Further investigation is required to study the occurrence of these strains in a wide range of RTs. Putative *β*-glucosidase gene knock-out studies compared with the wild type will aid in confirm the virulence of this variant strain in laboratory animals. Toxin A producing esculin hydrolysis-negative strains need to be taken into account in diagnostic clinical microbiology settings.

ACKNOWLEDGEMENTS

We are grateful to participants from PathWest Laboratory Medicine (WA) for collecting environmental samples and to Dr Papanin Putsathit and Dr Stacey Hong for their assistance with ribotyping. N.S. is funded by an Australian Government Research Training Program scholarship. D.R.K. is supported by a Fellowship from the National Health and Medical Research Council (APP1138257). K.I. is a recipient of the Mahidol Scholarship from Mahidol University, Thailand. Open access publishing facilitated by The University of Western Australia, as part of the Wiley - The University of Western Australia agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST

No conflict of interest was declared.

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How to cite this article: Shivaperumal, N, Knight, DR, Imwattana, K, Androga, GO, Chang, BJ, Riley, TV (2022) Esculin hydrolysis negative and TcdA-only producing strains of *Clostridium (Clostridioides) difficile* from the environment in Western Australia. *Journal of Applied Microbiology*. 133:1183–1196. https://doi.org/10.1111/jam.15500