PUTATIVE VIRULENCE FACTORS IDENTIFIED IN LARGE CLOSTRIDIAL TOXIN-NEGATIVE, BINARY TOXIN-PRODUCING *C. difficile* STRAINS

Androga GO[1,2], Knight DR[3], Hutton ML[4], Mileto SJ[4], James ML[4], Evans C[4], Foster NF[5], Lyras D[4], Riley TV[1, 2, 3, 6]

[1]The University of Western Australia, School of Biomedical Sciences, Western Australia, Australia; [2]PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Western Australia, Australia; [3] Murdoch University, School of Veterinary and Life Sciences, Western Australia, Australia; [4] Monash University, Department of Microbiology, Victoria, Australia; [5] Department of Health, Communicable Disease Control Directorate, Western Australia, Australia; [6] Edith Cowan University, School of Medical and Health Sciences, Western Australia, Australia

BACKGROUND AND AIMS

The relevance of large clostridial toxin-negative, binary toxin-producing [A$^-$B$^-$CDT$^+$] *C. difficile* strains in human infection is controversial. A$^-$B$^-$CDT$^+$ *C. difficile* strains [FIG 1] are considered clinically irrelevant despite their detection in symptomatic individuals and diarrhoeic animals[1]. Recently, we reported the presence of multiple AMR genes in these strains[1]. Here we investigate other putative virulence traits that may contribute to their role in idiopathic diarrhoea.

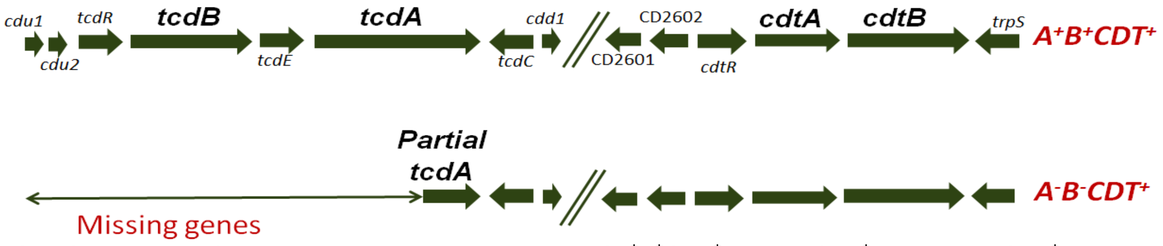

FIG 1. Comparison of pathogenicity and binary toxin loci of A$^+$B$^+$CDT$^+$ and A$^-$B$^-$CDT$^+$ strains. A$^-$B$^-$CDT$^+$ strains lack large clostridial toxin genes in general, however, some A$^-$B$^-$CDT$^+$ retain a non-functional fragment of the *tcdA* gene[2].

METHODS

- Phenotypic assays [motility, hydrolytic enzymes, *in vitro* and *in vivo* studies] were conducted on 148 A$^-$B$^-$CDT$^+$ *C. difficile* strains comprising 10 ribotypes [RTs 033, 238, 239, 288, 585, 586, QX143, QX444, QX521, QX629] [3,4].
- A subset of the *C. difficile* strains [*n*=53] were whole genome sequenced to identify genetic elements associated with virulence and survival [Table 1][5].

RESULTS

Motility

- Most [9/10] A$^-$B$^-$CDT$^+$ RTs tested were non-motile [RTs 033, 238, 288, 585, 586, QX143, QX444, QX521, QX629] including reference strain RT 078 [FIG 2]. A$^-$B$^-$CDT$^+$ RTs 033 and 288 had deletions in the F2 [glycosylation genes] and F3 [early-stage flagellar genes] regions of their flagellar operon while RTs 238, 585, 586, QX143, QX444, QX521, QX629 lacked the F2 region, retaining F1/F3 regions.
- The flagellar operon and the motility characteristic was conserved only in RT 239 and *C. difficile* references RTs 012 and 027 tested [FIG 2].
- The flagellin and flagella cap genes, *fliC* and *fliD*, involved in adherence and host colonisation, were conserved in all strains.

Extracellular Enzymes

- All A$^-$B$^-$CDT$^+$ *C. difficile* isolates produced at least 3 extracellular enzymes [deoxyribonuclease, esterase, mucinase], indicating that these are important extracellular proteins for these strains.
- Hyaluronidase and gelatinase were produced by 93/118 [RTs 033, 238, 288, 585, QX444, QX521] and 25/118 [RTs 033, 238, 288] A$^-$B$^-$CDT$^+$ *C. difficile* isolates respectively.
- No *C. difficile* isolates produced lecithinase, elastase or heparinase.

In vitro and *in vivo* assays

- Toxicity of the A$^-$B$^-$CDT$^+$ *C. difficile* strains was confirmed in Vero cells [FIG 3] but not reproduced *in vivo*.
- Mice infected with A$^-$B$^-$CDT$^+$ *C. difficile* strains all survived infection despite detection of high numbers of spores [10^7 CFU/g] in the faeces at either 24h or 96h post-infection [FIG 4].
- None had diarrhoea with the exception of mice infected with RT UK585 [ES 0618]. These mice had soft faeces/diarrhoea 24h post-infection and showed weight loss, however, they recovered.
- Despite successful colonisation by most of the strains [FIG 4], there was no evident disease phenotype. It is possible that the mouse model does not adequately demonstrate disease caused by A$^-$B$^-$CDT$^+$ *C. difficile* strains.

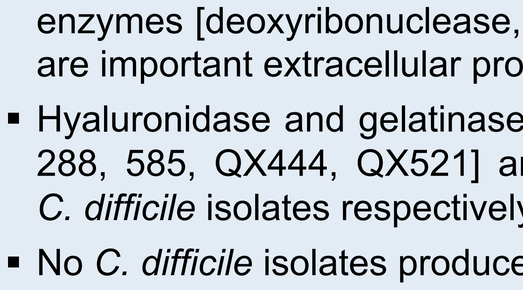

FIG 2. Demonstration of motile and non-motile *C. difficile* strains using reference isolates.

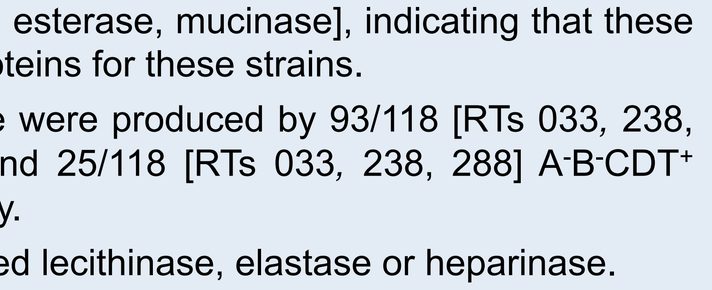

FIG 3. Cytopathic effect observed after exposing Vero cells to *C. difficile* filtrates.

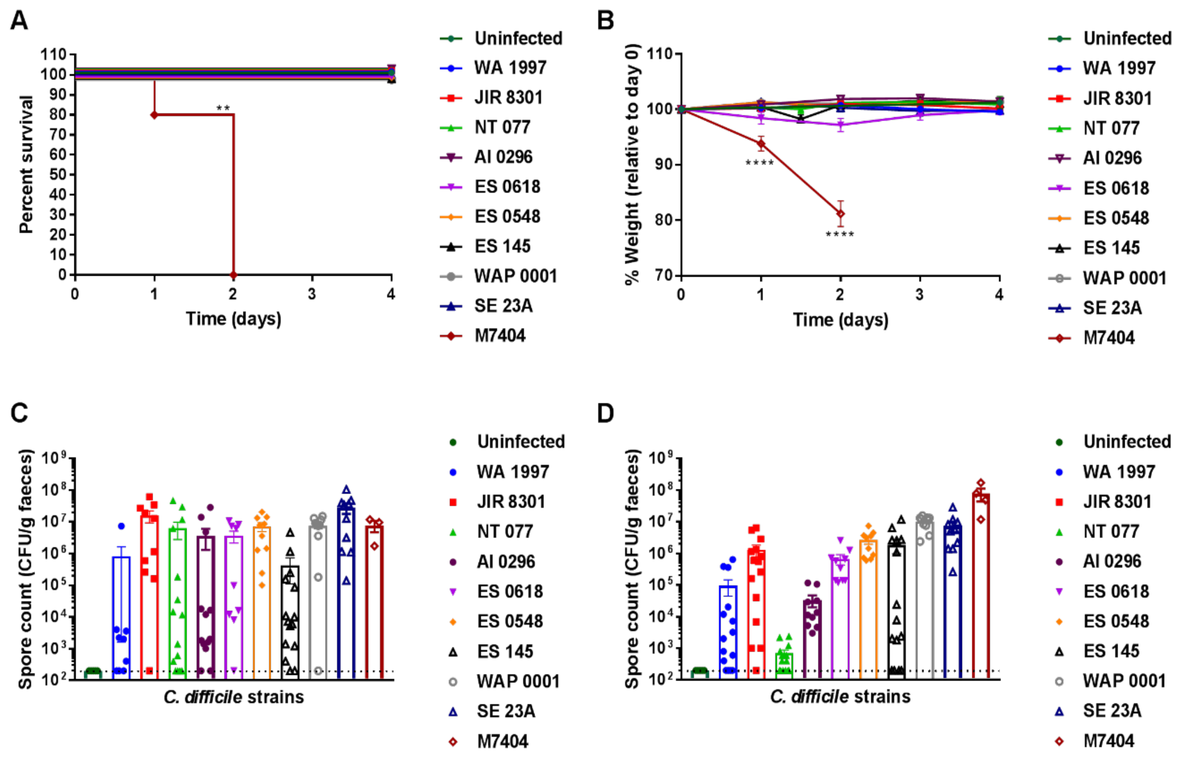

FIG 4. C57BL/6J mice infected with 10^5 spores of the *C. difficile* strains. [**A**] Survival graph; [**B**] Weight loss graph; [**C**] Spore count at 24h; [**D**] Spore count at 96h; **Positive control strain** [M7404 : A$^+$B$^+$CDT$^+$]; **A$^-$B$^-$CDT$^+$ strains** [WA 1997, JIR 8301, NT 077, ES 0618, ES 0548, ES 145, WAP 0001, SE 23A]

TABLE 1. Putative virulence proteins identified in A$^-$B$^-$CDT$^+$ *C. difficile* strains, also considered putative virulent traits in toxigenic strains.

Description	Gene identifier	Function	Ribotype, *n*
Toxin Production			
LytTR transcriptional regulator	CD2603[a]	Regulates binary toxin production[6]	All[b]
Ahesion, immunomodulation and motility			
Heat-shock inducible adhesin	*cwp66*	Mediates bacterial adherence to host cell[7,8]	All[b]
Cysteine protease	*cwp84*	Maturation and processing of the slpA layer, induces immune responses, breaks down gelatin, fibronectin, laminin and vitronectin proteins[7,8]	All[b]
Cell wall binding proteins	*cwp2, cwp5, cwp8, cwp11, cwp13, cwp25*	Involved in adhesion and assembly of a fully functional s-layer [6,8]	All[b]
Phase variable cell wall protein	*cwpV*	Bacterial aggregration, immune evasion[7,9]	All[b]
Major surface layer protein	*slpA*	Major contributor of host cell attachement and bacterial adherence[7,9]	All[b]
Collagen-binding proteins	CD2831[a]	Collagen-binding protein, recognition of extracellular matrix collagen[7]	All[b]
Capsule	CD3253[a], CD0775[a], CD2769[a]	Extracellular polysaccharide synthesis, immunomodulation[7]	All[b]
Haemagglutinin/ adhesin	CD0514[a]	Putative haemaglutinin[7]	All[b]
Fibronectin-binding protein	*fbpA*	Enables adherence to host cells[7]	All[b]
Flagellin proteins	*fliD, fliC*	Essential for fully functional flagella and involved in bacterial adherence to host cells[7]	All[b]
Putative typeIV pilus	CD3505[a]_CD3513[a]	Putative type IV pilus biosynthesis and function[7]	All[b]
Sortase	CD2718[a]	Class B sortase[7]	All[b]
Transmission [sporulation and germination]			
Stage 0 sporulation protein A	*spoA*	Sporulation transcription factor[7]	All[b]
Superoxide dismutase	*sodA*	Stress response[7]	All[b]
Spore coat proteins	*cotA, cotB, cotC, cotD, cotE, cotF, cotJB2, cotG*	Spore coat structure and morphogenesis[7]	All[b]
Cortex hydrolase	*sleC*	Essential for spore germination[10,11]	All[b]
Subtilisin-like serine protease	*cspC*	Protease that senses bile germinants and triggers activation of the hydrolase sleC[10,11].	All[b]
Antimicrobial resistance			
Aminoglycoside resistance	*aph3-III-sat4A-ant6-Ia*,	Aminoglycoside resistance[5,12]	RT033 [8/30]
	aph3-III-sat4A-npmA-ant6-Ia	Aminoglycoside resistance[5,12]	RT033 [1/30]
β-lactam resistance	*blaR, cme*	β-lactam resistance[5,12]	All[b]
Fluoroquinolone resistance	*gyrA/B*	Fluoroquinolone resistance[5,12]	RT033 [1/30]
Glycopeptide resistance	*VanB2 operon*	Vancomycin resistance[5,12]	RT033 [1/30]
Tetracycline resistance	*TetM*	Tetracycline resistance[5,12]	RT033 [1/30]
Adaptation and survival			
Cell lysis	CD1546[a]	Putative haemolysin like protein[7]	All[b]
p-hydroxyphenylacetate decarboxylase	*hpdBCA*	Catalyzes the decarboxylation of p-hydroxyphenylacetate, to yield the bacteriostatic compound, p-cresol[7]	All[b]
19-gene cluster hypothetical proteins	CD1906[a]_CD1926[a]	Ethanolamine degradation[7]	All[b]
Oxidoreductase	CD0065[a]	Converts primary bile acid [chenodeoxycholic acid] into a secondary acid [7-keto-litholic acid][7]	All[b]
Bile exclusion system	CD32150[a], CD32160[a]	Glycine/betaine ABC transporter ATP/substrate binding protein[7]	All[b]

[a]*C.difficile* Reference strain 630 [RT 012] [Genbank AM180355]. [b]53 A$^-$B$^-$CDT$^+$ *C. difficile* isolates [RTs 033, 238, 239, 288, 585, 586, QX143, QX444, QX521, QX629].

CONCLUSION

- We have confirmed toxin production and the presence of genes and/or proteins associated with survival, colonisation and disease pathogenesis in toxigenic *C. difficile* strains [2,5,6,7,8,9,10,11].
- This study provides the first in-depth analysis of A$^-$B$^-$CDT$^+$ *C. difficile* strains and highlights the need to further investigate their role in disease.

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