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

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BACKGROUND AND AIMS

The relevance of large clostridial toxin negative, binary toxin positive [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.

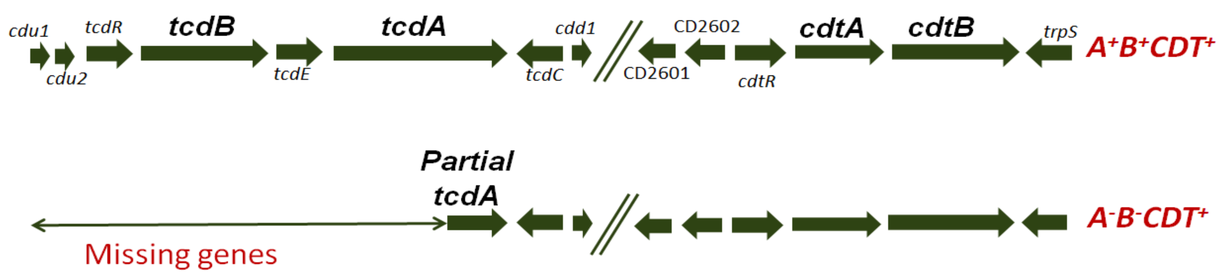

FIG 1. Comparison of pathogenicity and binary toxin loci of A⁺B⁺CDT⁺ and A⁻B⁻CDT⁺ strains. A⁻B⁻CDT⁺ strains lack large clostridial toxin genes in general, however, some A⁻B⁻CDT⁺ retain a non-functional fragment of *tcdA* gene.

METHODS

- Phenotypic assays were conducted on 148 A⁻B⁻CDT⁺ *C. difficile* strains comprising 10 ribotypes (RTs 033, 238, 239, 288, 585, 586, QX143, QX444, QX521, QX629), 53 of which were whole genome sequenced to identify genetic elements associated with virulence and survival[2].
- Pathogenicity assays were also conducted on A⁻B⁻CDT⁺ *C. difficile* strains using Vero cells and a C57BL/6J mouse model[3,4].

RESULTS

Motility

- As expected, 9/10 A⁻B⁻CDT⁺ RTs tested were non-motile [RTs 033, 238, 288, 585, 586, QX143, QX444, QX521, QX629]. RTs 033 and 288 had deletions in the F2 [glycosylation genes] and F3 [early-stage flagellar genes] regions of their flagellar operon while RTs 585, 586, QX143, QX444, QX521, QX629 lacked the F2 region, retaining F1/F3 regions.
- 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 major and important extracellular proteins for these strains.
- 93/118 [RTs 033, 238, 288, 585, QX444, QX521] and 25/118 [RTs 033, 238, 288] isolates produced hyaluronidase and gelatinase, respectively.
- None of the isolates produced lecithinase, elastase or heparinase hydrolytic enzymes.

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 strain QX146. These mice had soft faeces/diarrhoea 24h post-infection and showed weight loss, however, they recovered from the infection.
- It is possible that the mouse model does not adequately demonstrate disease caused by A⁻B⁻CDT⁺ *C. difficile* strains.

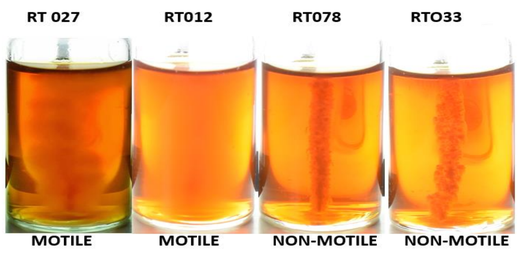

FIG 2. Demonstration of motile and non-motile *C. difficile* strains using reference isolates.

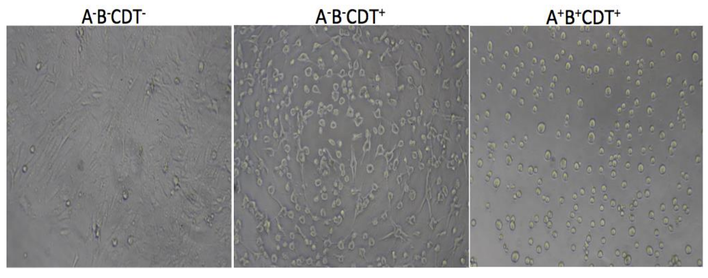

FIG 3. Cytopathic effect observed after exposing Vero cells to *C. difficile* filtrates.

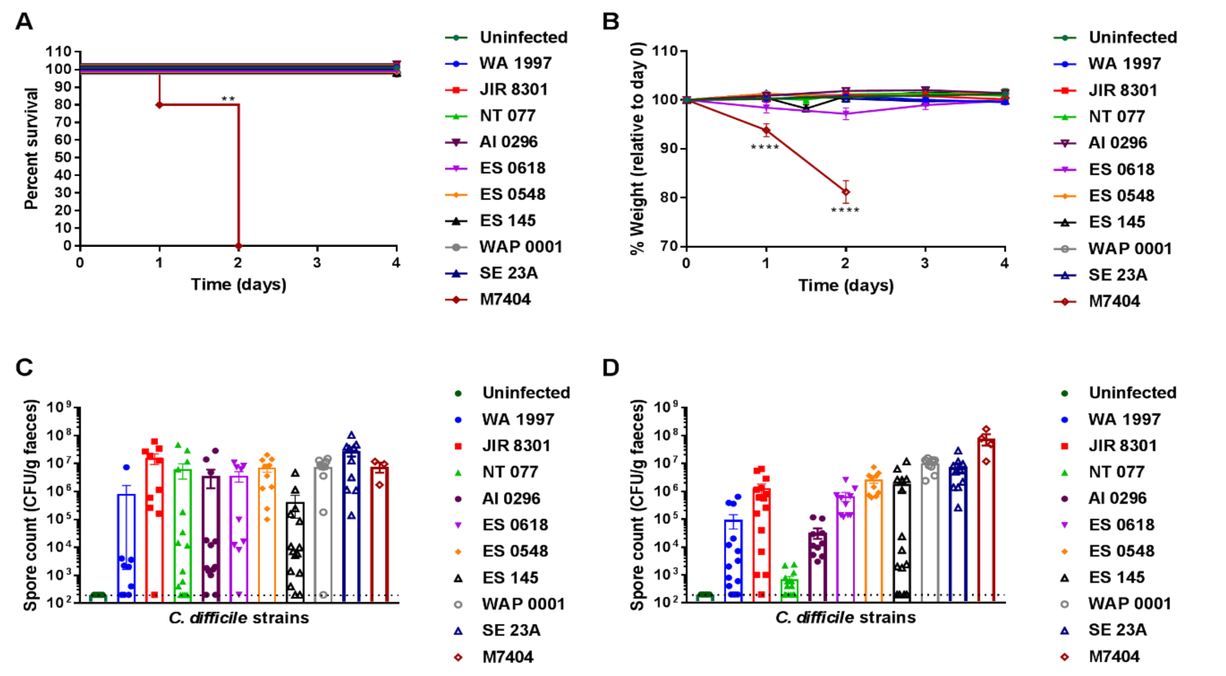

FIG 4. C57BL/6J mice infected with 10^5 spores of the *C. difficile* strains. (**A**) Survival graph; (**B**) Weight loss graph; (**C**) -Spore count at 24h; (**D**) -Spore count at 96h; **Positive control strain** (M7404 - A⁺B⁺CDT⁺); **A⁻B⁻CDT⁺ strains** (WA 1997, JIR 8301, NT 077, ES 0618, ES 0548, 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			
CDT Locus	CD2603[a]	Binary toxin production[5]	All A⁻B⁻CDT⁺ RTs tested[b]
Ahesion, immunomodulation and motility			
Heat-shock inducible adhesin	*cwp66*	Mediates bacterial adherence to host cell[6,7]	All A⁻B⁻CDT⁺ RTs tested[b]
Cysteine Protease	*cwp84*	Maturation and processing of the slpA layer, induces immune responses, breaks down gelatin, fibronectin, laminin and vitronectin proteins[6,7]	All A⁻B⁻CDT⁺ RTs tested[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 A⁻B⁻CDT⁺ RTs tested[b]
Phase variable cell wall protein	cwpV	Bacterial aggregration, immune evasion[6,8]	All A⁻B⁻CDT⁺ RTs tested[b]
Major surface layer protein	slpA	Major contributor of host cell attachement and bacterial adherence[6,8]	All A⁻B⁻CDT⁺ RTs tested[b]
Collagen-binding proteins	CD2831[a]	Collagen-binding protein, recognition of extracellular matrix collagen[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Capsule	CD3253[a], CD0775[a], CD2769[a]	Extracellular polysaccharide synthesis, immunomodulation[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Haemagglutinin/ adhesin	CD0514[a]	Putative haemaglutinin[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Fibronectin-binding protein	fbpA	Enables adherence to host cells[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Flagellin proteins	fliD, fliC	Essential for fully functional flagella and involved in bacterial adherence to host cells[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Putative TypeIV pilus	CD3505[a]_CD3513[a]	Putative type IV pilus biosynthesis and function[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Sortase	CD2718[a]	Class B sortase[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Transmission (sporulation and germination)			
Stage 0 sporulation protein A	spoA	Sporulation transcription factor[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Superoxide dismutase	sodA	Stress response[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Spore coat proteins	cotA, cotB, cotC, cotD, cotE, cotF, cotJB2, cotG	Spore coat structure and morphogenesis[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Cortex hydrolase	sleC	Essential for spore germination[9,10]	All A⁻B⁻CDT⁺ RTs tested[b]
Subtilisin-like serine protease	cspC	Protease that senses bile germinants and triggers activation of the hydrolase sleC[9,10].	All A⁻B⁻CDT⁺ RTs tested[b]
Antimicrobial resistance			
Aminoglycoside resistance	*aph3-III-sat4A-ant6-Ia*,	Aminoglycoside resistance[2,11]	RT033 (8/30)
	aph3-III-sat4A-npmA-ant6-Ia	Aminoglycoside resistance[2,11]	RT033 (1/30)
β-lactam resistance	*blaR, cme*	β-lactam resistance[2,11]	All A⁻B⁻CDT⁺ RTs tested[b]
Fluoroquinolone resistance	*gyrA/B*	Fluoroquinolone resistance[2,11]	RT033(1/30)
Glycopeptide resistance	*VanB2 operon*	vancomycin resistance[2,11]	RT033(1/30)
Tetracycline resistance	*TetM*	tetracycline resistance[2,11]	RT033(1/30)
Adaptation and survival			
Cell lysis	CD1546[a]	Putative haemolysin like protein[6]	All A⁻B⁻CDT⁺ RTs tested[b]
p-hydroxyphenylacetate decarboxylase	hpdBCA	Catalyzes the decarboxylation of p-hydroxyphenylacetate, to yield the bacteriostatic compound, p-cresol[6]	All A⁻B⁻CDT⁺ RTs tested[b]
19-gene cluster hypothetical proteins	CD1906[a]_CD1926[a]	Ethanolamine degradation[6]	All A⁻B⁻CDT⁺ RTs tested[b]
Oxidoreductase	CD0065[a]	Converts primary bile acid [chenodeoxycholic acid] into a secondary acid [7-keto-litholic acid][6]	All A⁻B⁻CDT⁺ RTs tested[b]
Bile exclusion system	CD32150[a], CD32160[a]	Glycine/betaine ABC transporter ATP/substrate binding protein[6]	All A⁻B⁻CDT⁺ RTs tested[b]

[a]*C.difficile* Reference strain 630 (Genbank AM180355). [b]53 A⁻B⁻CDT⁺ *C. difficile* isolates [RTs 033, 238, 288, 585, 586, QX143, QX444, QX521, QX629]

CONCLUSION

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 *C.difficile* disease.

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