Comparative genomic analysis of Clostridium difficile ribotype 027 strains including the newly sequenced strain NCKUH-21 isolated from a patient in Taiwan

Haruo Suzuki1,2, Masaru Tomita1,2, Pei-Jane Tsai3, Wen-Chien Ko4, Yuan-Pin Hung5,6, I-Hsiu Huang7* and Jenn-Wei Chen7*

Abstract

Background: Clostridium difficile is a Gram-positive anaerobe and the leading cause of antibiotic-associated diarrhea worldwide. The emergence of ribotype 027 (RT027) strains is associated with increased incidence of infection and mortality. To further understand the relationship between C. difficile NCKUH-21, a RT027 strain isolated from a patient in Taiwan, and other RT027 strains, we performed whole-genome shotgun sequencing on NCKUH-21 and comparative genomic analyses.

Results: The genome size, G+C content, and gene number for the NCKUH-21 strain were determined to be similar to those for other C. difficile strains. The core genome phylogeny indicated that the five RT027 strains R20291, CD196, NCKUH-21, BI1, and 200785 formed a clade. A pathogenicity locus, tcdR-tcdB-tcdE-orf-tcdA-tcdC, was conserved in the genome. A genomic region highly similar to the Clostridium phage ϕCD38-2 was present in the NCKUH-21 strain but absent in the other RT027 strains and designated as the prophage ϕNCKUH-21. The prophage ϕNCKUH-21 genes were significantly higher in G+C content than the other genes in the NCKUH-21 genome, indicating that the prophage does not match the base composition of the host genome.

Conclusions: This is the first whole-genome analysis of a RT027 C. difficile strain isolated from Taiwan. Due to the high identity with ϕCD38-2, the prophage identified in the NCKUH-21 genome has the potential to regulate toxin production. These results provide important information for understanding the pathogenicity of RT027 C. difficile in Taiwan.

Keywords: Clostridium difficile, Ribotype 027 strain NCKUH-21, Genome, Phylogeny, Prophage, Horizontal transfer

Background

Clostridium difficile is a Gram-positive, endospore-forming obligate anaerobe and the current leading cause of antibiotic-associated diarrhea (AAD) within hospital settings worldwide [1]. Estimates have revealed that C. difficile infections (CDI) are responsible for 15–25% of all AAD cases [2]. Onset of CDI can be engendered by disruption of the hosts’ gut microbiota by broad-spectrum antibiotic treatments. Aging, prolonged stay in healthcare settings, and proton-pump inhibitor use all contribute to increased risk of CDI [3]. Although C. difficile has been characterized for decades, it first gained prominence in 2003 when an outbreak in North America was found to be caused by a strain with toxin hyperproduction capabilities [4]. The rapid spread of C. difficile NAP1/BI/027 strain (PCR ribotype 027 or RT027), which is the same strain characterized with different methods has resulted in outbreaks worldwide, although cases in Asia and Latin America were less reported compared with Europe and North America.
According to a previous case report, NCKUH-21 is the strain isolated from the first severe RT027 CDI in Taiwan, and it contains a deletion of 18 base pairs and a truncated mutation (D117A) in tcdC [5]. To further understand the relationship between NCKUH-21 and other RT027 strains including historic strains and hypervirulent strains, we determined the genome sequence of the *Clostridium difficile* strain NCKUH-21 (the accession numbers: BDSN01000001–BDSN01000094) and compared it with other sequenced RT027 strains. We assessed the presence of virulence and antibiotic resistance genes for the NCKUH-21 genome. We also compared the genome sequences of the NCKUH-21 strain with its close relatives to investigate the genome synteny, reconstruct the phylogenetic tree, and identify NCKUH-21 strain-specific genes.

**Methods**

Genome sequencing, assembly, and annotation for the strain NCKUH-21, as well as comparative genomics of nine *C. difficile* strains (Table 1), were performed as described in Additional file 1: Materials and methods.

**Quality assurance**

Genomic DNAs were purified from a pure culture of a single bacterial isolate of NCKUH-21. A BLAST search against a nonredundant database revealed no potential contamination of the genomic libraries.

**Results and discussion**

**Genomic features**

Illumina MiSeq sequencing was performed to determine the genome sequence of the *C. difficile* strain NCKUH-21. The de novo assembly contained 94 contigs of length 4,217,149 bp, with a G+C content of 28.4% with sequencing coverage of 1611×. Genome annotation yielded a total of 3810 protein-coding sequences (CDSs).

Among the *C. difficile* strains analyzed in this paper, the genome size (Mb) ranged from 4.05 to 4.46, G+C content ranged from 28.4 to 29.2%, and CDS number ranged from 3485 to 4128 (Table 1). The general genomic features for the NCKUH-21 strain were thus similar to those of the other *C. difficile* strains.

**Phylogeny**

*Clostridium difficile* strains with the same PCR ribotype were reported to cluster together in the phylogenetic trees for the conserved genes [6]. The Roary pipeline produced a total of 8775 homologous groups of genes (“pan-genome”), of which 69 were shared by all the strains used in this study (“core-genome”). The core genome phylogeny indicated that the RT027 strains (R20291, CD196, NCKUH-21, BI1, and 2007855) formed a monophyletic group or clade, joined by the Z31 and 630 strains, followed by the M68 strain, and finally the M120 strain (Fig. 1).

**Synteny**

The Mauve Contig Mover (http://darlinglab.org/mauve/user-guide/reordering.html) was used to reorder the contigs of NCKUH-21 relative to the complete genome of *C. difficile* CD196. The genomes of the nine *C. difficile* strains were aligned using progressiveMauve, and this alignment was visualized using genoPlotR to investigate genomic rearrangement (Fig. 2). The genome synteny was determined to be conserved among all but one of the strains. An exception was the Z31 strain with large-scale genomic rearrangement, which had not been previously reported [7].

**Table 1 Analysis of the genomic features of Clostridium strains**

| Organism name | Size (bp) | %G+C | CDS | Source |
|---------------|-----------|-------|-----|--------|
| *C. difficile* R20291 | 4,191,339 | 28.8 | 3543 | An epidemic strain, UK, 2006 |
| *C. difficile* CD196 | 4,110,554 | 28.6 | 3485 | A patient with CDI, France, 1985 |
| *C. difficile* NCKUH-21 | 4,217,149 | 28.4 | 3810 | A patient with severe PMC, Taiwan, 2014 |
| *C. difficile* BI1 | 4,464,700 | 28.4 | 4101 | A human strain, USA, 1988 |
| *C. difficile* 2007855 | 4,179,867 | 28.7 | 3811 | A bovine strain, USA, 2007 |
| *C. difficile* Z31 | 4,298,263 | 29.2 | 4128 | A canine NTCD strain, Brazil, 2009 |
| *C. difficile* CD630 | 4,298,133 | 29.1 | 3908 | A patient with severe PMC, Switzerland, 1982 |
| *C. difficile* M68 | 4,308,325 | 28.9 | 3870 | A human strain, Ireland, 2006 |
| *C. difficile* M120 | 4,047,729 | 28.7 | 3634 | A human strain, UK, 2007 |
| *C. mangenotii* LM2 | 3,023,790 | 31.6 | 2808 | A reference genome from the rumen microbiome |

%G+C= 100×(G+C)/(A+T+G+C)

CDS number of protein-coding DNA sequences, PMC pseudomembrane colitis, NTCD non-toxigenic *C. difficile*
Antibiotic resistance and virulence genes
Antibiotic resistance and virulence genes were searched using ABRicate. Homologous DNA sequences for the binary toxin genes *cdtA* and *cdtB* listed in the Virulence Factors Database (accessions of AAF81760 and AAF81761, respectively) were detected in the NCKUH-21 genome [8]. Homologous DNA sequences for the antibiotic resistance genes *cdeA*, *vanRG*, and *vanG* listed in the Comprehensive Antibiotic Resistance Database (accessions of AJ574887.1:371–1697, DQ212986:2259–2967, and DQ212986:5985–7035, respectively) were detected in the NCKUH-21 genome. Although NCKUH-21 showed the genetic potential for becoming resistant to antibiotics, this strain was shown to be susceptible to moxifloxacin (minimum inhibitory concentration 0.5 μg/mL), metronidazole (0.094 μg/mL), and vancomycin (0.5 μg/mL) [5].

The genetic organization of the pathogenicity locus (PaLoc) of the CD630 strain is *tcdR*-*tcdB*-*tcdE*-orf*-tcdA*-*tcdC* (locus_tag: CD630_06590, CD630_06600, CD630_06610, CD630_06620, CD630_06630, and CD630_06640) [9]. The gene order was conserved in the NCKUH-21 genome (the accession number: BDSN01000011; locus_tag: NCKUH21_00647, NCKUH21_00648, NCKUH21_00649, NCKUH21_00650, NCKUH21_00651, and NCKUH21_00652). Moreover, another sequence similar to *tcdE* (CD630_06610) was found in the NCKUH-21 genome (locus_tag: NCKUH21_00647) with 83% amino acid identity. The genes *tcdB* and *tcdA* encoding Toxin B and Toxin A (locus_tag: CD630_06600 and CD630_06630; 2366 and 2710 amino acids in length), respectively, of the CD630 PaLoc were determined to be homologous with 48% amino acid identity; additionally, these two genes partly matched a sequence encoding “N-acetylmuramoyl-L-alanine amidase LytC” (the accession number: BDSN01000021; locus_tag: NCKUH21_02692; 644 amino acids in length) in the NCKUH-21 genome with 177 and 226 alignment length and 32 and 34% amino acid identity values, respectively. The PaLoc gene homologues may contribute to the virulence and pathogenicity for the *C. difficile* strain NCKUH-21.

NCKUH-21 strain-specific genes
To identify NCKUH-21 strain-specific genes, we searched the NCKUH-21 strain's protein homologues in the genome sequences of all *C. difficile* strains by using the gene screen method with TBLASTN in the large-scale blast score ratio (LS-BSR) pipeline. Of the 3810 protein-coding genes identified in NCKUH-21, 3579 were
conserved in all the other RT027 strains (R20291, CD196, B11, and 2007855), and 2832 were conserved in all the C. difficile strains used in this study. Among the strains, the largest numbers of NCKUH-21 genes were conserved in the RT027 strains (R20291, CD196, B11, and 2007855), ranging from 3592 to 3655, followed by other C. difficile strains (Z31, 630, M68, and M120), ranging from 3153 to 3431, and finally the outgroup LM2 (761).

A total of 140 protein-coding genes were present in the NCKUH-21 strain but absent in the other strains (Additional file 2: Table S1). The NCKUH-21 strain-specific genes could have been gained on the branch leading to the NCKUH-21 strain, and they could thus be linked to its specific phenotype (e.g., virulence and pathogenicity). Of the 140 NCKUH-21 strain-specific genes, 50 were encoded on the 40,525-bp-long contig sequence of the NCKUH-21 genome (the accession number: BDSN01000034), which showed a 99% identity match to the Clostridium phage ϕCD38-2 (GenBank accession: HM568888). The genomic region highly similar to the Clostridium phage ϕCD38-2 was designated as the prophage ϕNCKUH-21.

**Prophage ϕNCKUH-21**
The prophage ϕNCKUH-21 detected in the draft genome for the C. difficile strain NCKUH-21 was further confirmed by phage induction examination and electron microscope imaging (data not shown). A previous study suggested that lysogenic ϕCD38-2 replicates as a circular plasmid and boosts toxin production in C. difficile [10]. The high sequence identity between ϕNCKUH-21 and ϕCD38-2 suggests that these prophages have a similar role in C. difficile.

Reports have revealed that bacterial phages tend to be lower in G+C content than their hosts and that viruses match the G+C content of their hosts [11, 12], including the C. difficile bacteriophage ϕCD119 [13]. Base composition statistics for the NCKUH-21 genes were calculated as the relative frequency of G+C at third codon positions (GC3). The median GC3 value for the prophage ϕNCKUH-21 genes (0.21) was higher than that for the other genes (0.14) in the NCKUH-21 genome. A Wilcoxon rank sum test, which compared the GC3 values between the two groups of genes, was highly significant (P < 2.2e−16). This suggests that the prophage ϕNCKUH-21 does not match the base composition of the host genome and may thus have been acquired by horizontal transfer based on the hypothesis of genome amelioration [14].

**Concluding remarks**
From 2013 to 2014, three RT027 C. difficile strains were isolated from patients in Taiwan [5, 15, 16]. Among them, NCKUH-21 is the first strain to have a whole-genome sequence for genome comparison. Whether the other two RT027 isolates also carry a complete prophage, what their phylogenetic relation with NCKUH-21 is, and what the relative toxin production level is between the three isolates are all topics for further research.

**Additional files**

**Additional file 1.** Materials and methods.

**Additional file 2: Table S1.** Data for Clostridium difficile strain NCKUH-21 genes. The columns are as follows: locus_tag, length in amino acids (Laa), G+C content at the third codon positions (GC3), binary number (1 or 0) indicating whether the gene is NCKUH-21 strain-specific (StrainSpecific), gene and product names, and the most similar sequence annotation in the UniRef90 database (FASTA header and organism name).

**Authors’ contributions**
HS conducted the bioinformatics analyses and drafted the manuscript. MT managed bioinformatics environments and helped write the manuscript. JWC performed the laboratory experiments and wrote the manuscript. IHH provided experimental suggestions and wrote the manuscript. PJT, WCK, and YPH provided the isolate and clinical characterizations. All authors read and approved the final manuscript.

**Author details**
1 Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata, Japan. 2 Faculty of Environment and Information Studies, Keio University, Fujisawa, Kanagawa, Japan. 3 Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University, Tainan, Taiwan. 4 Department of Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan. 5 Department of Internal Medicine, Tainan Hospital, Ministry of Health & Welfare, Tainan, Taiwan. 6 Department of Internal Medicine, National Cheng Kung University Hospital, Tainan, Taiwan. 7 Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, 1 University Road, Tainan 70101, Taiwan.

**Acknowledgements**
Computational resources were provided by the Data Integration and Analysis Facility, National Institute for Basic Biology, Japan.

**Competing interests**
The authors declare that they have no competing interests.

**Availability of data and materials**
Nucleotide sequence accession numbers: The whole-genome shotgun sequencing data have been deposited in DDBJ/EMBL/GenBank under the accession numbers BDSN01000001–BDSN01000094 (94 entries).

**Consent for publication**
Not applicable.

**Ethics approval and consent to participate**
Not applicable.

**Funding**
This work was supported in part by research funding from Keio University and from Yamagata Prefecture and Tsuruoka City, Japan, and Ministry of Science and Technology, Taiwan, Grants (103-2320-B-006-028-MY2 to JC, 106-2633-B-006-002- to IH).

**Publisher’s Note**
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
References

1. Ananthakrishnan AN. *Clostridium difficile* infection: epidemiology, risk factors and management. Nat Rev Gastroenterol Hepatol. 2011;8(1):17–26.

2. Bartlett JG, Gerding DN. Clinical recognition and diagnosis of *Clostridium difficile* infection. Clin Infect Dis. 2008;46(Suppl 1):S12–8.

3. Jump RL. *Clostridium difficile* infection in older adults. Aging Health. 2013;9(4):403–14.

4. O’Connor JR, Johnson S, Gerding DN. *Clostridium difficile* infection caused by the epidemic B/NAP1/027 strain. Gastroenterology. 2009;136(6):1913–24.

5. Hung YP, Cia CT, Tsai BY, Chen PC, Lin HJ, Liu HC, Lee JC, Wu YH, Tsai PJ, Ko WC. The first case of severe *Clostridium difficile* ribotype 027 infection in Taiwan. J Infect. 2015;70(1):98–101.

6. Kurka H, Ehrenreich A, Ludwig W, Monot M, Rupnik M, Barbut F, Indra A, Dupuy B, Liebl W. Sequence similarity of *Clostridium difficile* strains by analysis of conserved genes and genome content is reflected by their ribotype affiliation. PLoS ONE. 2014;9(1):e86535.

7. Pereira FL, Oliveira Junior CA, Silva ROS, Dorella FA, Carvalho AF, Almeida GMF, Leal CAG, Lobato FCF, Figueiredo HCP. Complete genome sequence of Peptoclostridium difficile strain Z31. Gut Pathog. 2016;8:11.

8. Gerding DN, Johnson S, Rupnik M, Aktones K. *Clostridium difficile* binary toxin CDT: mechanism, epidemiology, and potential clinical importance. Gut Microbes. 2014;5(1):15–27.

9. Monot M, Eckert C, Lemire A, Hamiot A, Dubois T, Tesser C, Dumoulard B, Hamel B, Pettit A, Lalande V, et al. *Clostridium difficile*: new insights into the evolution of the pathogenicity locus. Sci Rep. 2015;5:15023.

10. Sekulovic O, Meessen-Pinard M, Fortier LC. Prophage-stimulated toxin production in *Clostridium difficile* NAP1/027 lysogens. J Bacteriol. 2011;193(11):2726–34.

11. Rocha EP, Danchin A. Base composition bias might result from competition for metabolic resources. Trends Genet. 2002;18(6):291–4.

12. Cardinale DJ, Duffy S. Single-stranded genomic architecture constrains optimal codon usage. Bacteriophage. 2011;1(4):219–24.

13. Govind R, Frilick JA, Rolfe RD. Genomic organization and molecular characterization of *Clostridium difficile* bacteriophage PhiCD119. J Bacteriol. 2006;188(7):2568–77.

14. Lawrence JG, Ochman H. Amelioration of bacterial genomes: rates of change and exchange. J Mol Evol. 1997;44(4):383–97.

15. Liao TL, Lin CF, Chiou CS, Shen GH, Wang J. *Clostridium difficile* PCR ribotype 027 emerges in Taiwan. Jpn J Infect Dis. 2015;68(4):338–40.

16. Lai MJ, Chiueh TS, Huang ZY, Lin JC. The first *Clostridium difficile* ribotype 027 strain isolated in Taiwan. J Formos Med Assoc. 2016;115(3):210–2.