

# Limitations of Ribotyping as Genotyping Method for *Corynebacterium ulcerans*

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We conducted molecular typing of a *Corynebacterium ulcerans* isolate from a woman who died in Japan in 2016. Genomic DNA modification might have affected the isolate's ribotyping profile. Multilocus sequence typing results (sequence type 337) were more accurate. Whole-genome sequencing had greater ability to discriminate lineages at high resolution.

*Corynebacterium ulcerans* is a zoonotic pathogen that causes an illness categorized in World Health Organization documents as diphtheria (1). Genotyping methods such as ribotyping, multilocus sequence typing (MLST), and whole-genome sequencing are used to classify isolates. During the 1990s and early 2000s, the standard molecular typing method of *Corynebacterium diphtheriae* was conventional ribotyping (2,3). Ribotyping is also used to classify *C. ulcerans* (4) and compare isolates (5–9). Today, the standard method is MLST because of its objectivity and reproducibility (8,10). We sequenced 3 isolates of *C. ulcerans* from patients in Japan to analyze the accuracy of conventional ribotyping, MLST, and whole-genome sequencing.

## The Study

In 2016, a 66-year-old woman in Fukuoka, Japan, died of a diphtheria-like disease. Otsuji et al. isolated toxigenic *C. ulcerans* from the patient's tracheal pseudomembrane and blood (6). We analyzed the isolate (FH2016-1) from the pseudomembrane alongside the first (11) and second (5) *C. ulcerans* isolates taken from patients in Japan; the first isolate (0102) was taken in 2001 and the second isolate (0211) in 2002.

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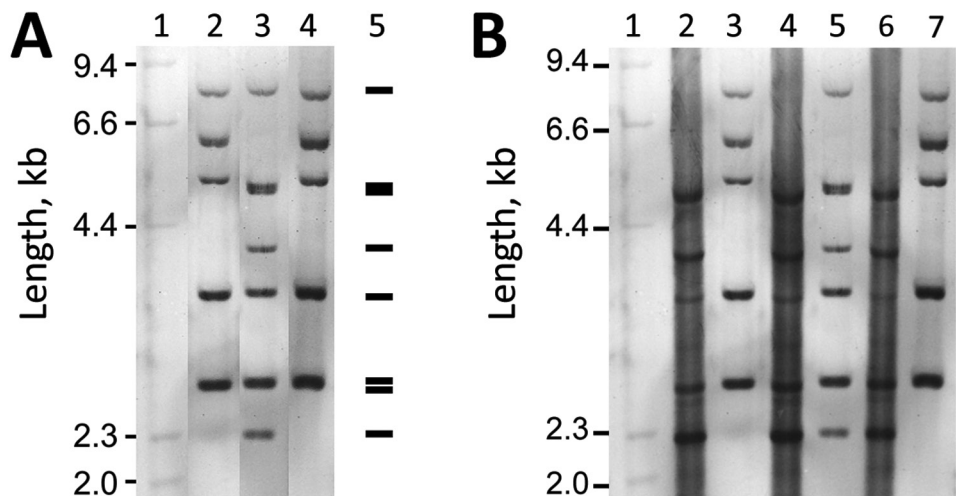
Strains 0102 and 0211 (named for the initial isolates taken in 2001 and 2002) are the 2 major ribotypes of *C. ulcerans* in Japan. Our conventional ribotyping of the isolates found the pattern obtained from FH2016-1 was indistinguishable from that of 0102, indicating that FH2016-1 belongs to strain 0102 (Figure 1, panel A).

We also whole-genome sequenced strains FH2016-1 and 0211 using the NextSeq500 Illumina (for strain FH2016-1 [Illumina, <https://www.illumina.com>]), Illumina GAII (for strain 0211 [Illumina]), ABI 3730xl (Thermo Fisher, <https://www.thermofisher.com>), and PacBio Sequel (Pacific Biosciences of California, Inc., <https://www.pacb.com>) sequencers, followed by de novo assembly. We deposited complete sequences and assembly methods in GenBank under accession nos. AP019663 (strain FH2016-1) and AP019662 (strain 0211). Using these sequences and the previously published genome sequence (12) of strain 0102 (GenBank accession no. AP012284), we conducted in silico ribotyping of *Bst*EII-digested fragments that hybridized with OligoMix5 probes, producing a predicted pattern for each sequence (13). The predicted patterns of all 3 strains matched the conventional ribotype pattern of strain 0211. However, the conventional ribotyping patterns of strains FH2016-1 and 0102 did not match the in silico-predicted ribotype pattern (Figure 1, panel A).

The discrepancy between the conventional and in silico-predicted patterns is caused by impaired restriction digestion at specific *Bst*EII sites. In these strains, the conventional (modified) ribotype pattern differed from the in silico-predicted (unmodified) ribotype pattern by a shift of 4 fragments (Appendix Figure 1, panel A, <https://wwwnc.cdc.gov/EID/article/26/10/20-0086-App1.pdf>). For example, in silico typing predicted that 3 *Bst*EII sites would be digested at nt 770,000 of strain FH2016-1. PacBio modification analysis revealed that 1 of these sites might have been modified

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**Figure 1.** Alteration of ribotyping patterns by genomic DNA modification of *Corynebacterium ulcerans* strains 0102, 0211, and FH2016-1, Japan, 2001–2016. Ribotyping was performed as described previously (4,11). *Hind*III-digested, digoxigenin-labeled  $\lambda$  phage DNA segments were used as length markers. A) Conventional ribotyping patterns of strains 0102, 0211, and FH2016-1. 1,  $\lambda$ *Hind*III; 2, 0102; 3, 0211; 4, FH2016-1; 5, Pattern predicted by in silico typing. B) Ribotyping patterns of genomic DNA and whole-genome amplified DNA as substrates. 1,  $\lambda$ *Hind*III; 2, 0102 WGA; 3, 0102 native; 4, 0211 WGA; 5, 0211 native; 6, FH2016-1 WGA; 7, FH2016-1 native. The label “WGA” indicates



whole-genome amplified DNA as a substrate; “native” indicates genomic DNA. WGA (unmodified) DNA of the 3 strains show identical patterns. The pattern matches that of native 0211 (unmodified genomic DNA). In contrast, native FH2016-1 and 0102 are modified and show different patterns from their WGA counterparts.

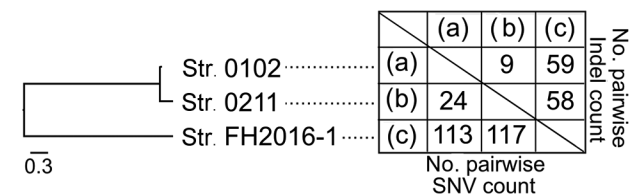
(Appendix Figure 1, panel B). *Bst*EII is sensitive to methylation and other types of DNA modification (14). Thus, the difference in restriction fragment patterns was closely related to the nucleotide modifications within *Bst*EII recognition sites (Appendix Figure 1, panel B). Other *Bst*EII sites also might have been modified, resulting in the 4-fragment shift. Accordingly, we did not observe this shift in ribotypes of unmodified DNA substrate prepared by whole-genome amplification of the 3 strains (15) (Figure 1, panel B). The patterns of unmodified DNA matched the pattern of strain 0211 (Figure 1, panel B) and the in silico-predicted pattern (Figure 1, panel A). The  $\geq 6.1$ -kb bands seen in “native” lanes were not visible in whole-genome amplification lanes, potentially because of the failure of whole-genome amplification to generate large fragments. These results indicate that ribotyping patterns might be substantially affected by DNA modification.

The sequences of strains FH2016-1, 0102, and 0211 were highly homologous. For example, they shared complete sequence identity (data not shown) for a structural gene (locus tag CULCFH20161\_03390) encoding a DNA methylase. However, we observed small differences in their genomes (Table; Figure 2; Appendix Table 1). We expected factors contributing to genomic DNA modification to be common between strains FH2016-1 and 0102, but not 0211. Scanning the genomes of the 3 strains for such factors resulted in 15 candidate open reading frames (ORFs) (Table). None of these ORFs contained motifs related to DNA methylation; however, these ORFs might still contribute to

DNA modification of other gene products. The nature of the modification(s) remains unknown.

Conventional ribotyping (Figure 1, panel A) showed that strains FH2016-1 and 0102 were closely related. However, comparison of 30 genome sequences of strains from around the world (Appendix Table 2, Figure 2) revealed that all 3 strains from Japan belong to a single phylogenetic cluster and sequence type (ST) 337. Whether the 3 isolates represent the entire population of *C. ulcerans* in Japan is unclear. However, more than half the isolates we have analyzed ( $\approx 20$ ) are ST337 (M. Iwaki and A. Yamamoto, unpub. data), suggesting a small amount of genetic diversity among the *C. ulcerans* population in Japan.

Close-up view of the phylogenetic tree showed that these strains from Japan divided into 2 different lineages. At most, 117 single nucleotide variations and 59 insertions/deletions existed between any 2 strains (Figure 2). Although this result indicated low variability



**Figure 2.** Genetic similarity among 3 selected strains of *Corynebacterium ulcerans*, Japan, 2001–2016. Strain 0102 is represented by (a), strain 0211 by (b), and strain FH2016-1 by (c). Numbers of SNVs and indels between strains are shown. A phylogenetic tree generated by SNV data are shown on the left. Indel, insertion/deletion; SNV, single-nucleotide variation.

among the 3 strains, it also showed that strain FH2016-1 was genetically distinct from 0102 and 0211 (Figure 2). Thus, the genome sequence analysis indicated that conventional ribotyping did not reflect lineage accurately and resulted in a misleading classification of these specimens. In contrast, MLST, which is now the preferred method of molecular typing (8,10), provided more accurate results. We queried the genomic sequences of the 3 strains on the PubMLST website (<https://pubmlst.org>) and analyzed them at 7 alleles (*atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA*, and *rpoB*). The same sequence type (ST337) was assigned to all 3 strains, reflecting the low genetic variability among these strains.

## Conclusions

Our study of 3 strains of *C. ulcerans* showed that conventional ribotyping is less accurate than other methods of phylogenetic analysis. In comparison, MLST is less erroneous, and whole-genome sequencing produces results with greater resolution than those of conventional ribotyping. MLST produced results with lower resolution than whole-genome sequencing while maintaining a high level of accuracy. MLST and whole-genome sequencing improve the accuracy and efficiency of phylogenetic analysis of *C. ulcerans*.

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