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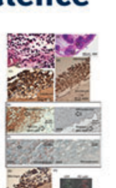

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Wiep Klaas Smits

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EDITORIAL

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SNP-ing out the differences: Investigating differences between *Clostridium difficile* lab strains

Wiep Klaas Smits

Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands

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Clostridium difficile is an Gram positive enteropathogen that can cause opportunistic infections resulting in colitis.[1,2] It is predominantly associated with antibiotic treatment, but it is increasingly recognized as the causative agent of symptoms in patients that lack this risk factor.[1,3] Its classification as an urgent antibiotic resistance threat by the US Centers for Disease Control and Prevention[4] is based on the fact that the pathogen affects an already vulnerable population that is treated by antibiotics for other infections or prophylactically, rather than resistance of *C. difficile* against clinically used antibiotics, which is limited.[1] *C. difficile* can be identified in livestock and companion animals and it has been shown that strains from animal and human reservoirs are identical, suggesting a clear zoonotic potential.[5-7]

Initially identified as *Bacillus difficilis* as part of the microbiome of healthy infants,[8] it gained notoriety as *Clostridium difficile* and the disease it causes is generally referred to as *Clostridium difficile* infection (CDI). Genomic analyses however indicated that *C. difficile* should be placed in the family *Peptostreptococcaceae* rather than *Clostridiaceae*, and to reflect this the name *Peptoclostridium difficile* was proposed.[9] Though this was unilaterally adopted by the National Center for Biotechnology Information (NCBI), the proposal lacked a formal definition of the type species and the name was not widely adopted by the community. A formal reclassification was published in 2016[10] and the new nomenclature *Clostridioides difficile* allows the continued use of CDI, as well as the colloquialism Cdiff.

The symptoms of CDI are the ultimate result of toxins produced by the *C. difficile* bacteria.[11] The genes encoding these toxins are located on a mobile pathogenicity locus.[12-14] Indeed, strains lacking the pathogenicity island are non-toxigenic. Most pathogenic *C. difficile* strains encode 2 high molecular weight toxins, TcdA and TcdB, and the relative contribution of these toxins to pathogenesis has been subject of controversy.[15-18] Similarly, conflicting findings have been reported with respect to the function of other proteins (TcdC, TcdE) encoded on the pathogenicity locus.[1,19] Further, certain *C. difficile* strains encode a binary toxin that contributes to pathogenesis.[1,11,19-21] Overall, virulence, fitness and transmissibility of the pathogen appear to be multifactorial.[19,22,23]

In this issue of *Virulence*, Collery and coworkers attempt to identify a possible cause of the discrepancies observed between various laboratories studying *C. difficile*.[24] To appreciate the intricacies of this work, it is necessary to understand the background of the strains that were investigated. The first strain of *C. difficile* to be sequenced was isolated from a patient suffering pseudomembranous colitis and was responsible for an outbreak of CDI in the hospital in Zürich.[25,26] This strain, called 630, demonstrated transferable resistance to the antimicrobial erythromycin and is also resistant to several other drugs.[25,26] The strain was redistributed to several other labs, and the annotation of the genome sequence has been updated several times.[27,28] In order to facilitate genetic studies on *C. difficile*, 2 groups independently derived an erythromycin sensitive strain by serial culturing on non-selective media: the Mullany laboratory (University College London, London, UK) generated strain 630Δ*erm*,[29] and the Rood laboratory (Monash University, Victoria, Australia) generated 630E (also known as JIR8094).[30] These strains – harboring an identical 2.4 kb deletion in the mobile element Tn*5398*,[31-33] allowed the use of the *ermB* gene (conferring erythromycin resistance) as a selectable marker in *C. difficile*. Both 630E and 630Δ*erm* were provided to other laboratories. Notably, both 630 and 630Δ*erm* have been deposited

CONTACT Wiep Klaas Smits w.k.smits@lumc.nl Department of Medical Microbiology, Leiden University Medical Center, PO Box 9600 2300RC, Leiden, The Netherlands.

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directly or indirectly in various culture collections [ATCC (https://www.atcc.org), NCTC (https://www.phe-culturecollections.org.uk) and DSMZ (https://www.dsmz.de)] which – in turn – provide bacterial strains to other scientists (Fig. 1). Whereas the DNA from the 2006 genome sequence of strain 630 came from the Mullany laboratory,[25] DNA from 2 independent resequencing projects was derived from the isolates banked by the NCTC (NCTC3000 Project, https://www.phe-culturecollections.org.uk/collections/nctc-3000-project.aspx) and the DSMZ.[34] The latter genome sequence shows some peculiar features, including the apparent loss (i.e. not detected in their analyses) of plasmid pCD630 and transposon Tn*5397* and acquisition of an additional rRNA cluster, that seem to suggest extensive sub-culturing. Of note, the DSMZ strain was obtained from the NCTC, that in turn received its isolate from the Mullany laboratory. A single study has reported a complete genome sequence for strain 630Δ*erm*[33] prior to the study of Collery and coworkers.[24] Strikingly, the authors identified many more differences from strain 630 than the deletion of an *ermB* gene in Tn*5398*, including a transposition of the conjugative transposon CTn*5* and an additional rRNA cluster, similar to the resequenced 630 strain.[34] Thus, despite a common ancestry, the strains differ vastly.

The study of Collery and coworkers is noteworthy for several reasons. First, it represents a multi-laboratory effort to determine if differences reported in literature are due to the different 630 derivatives used (630Δ*erm*

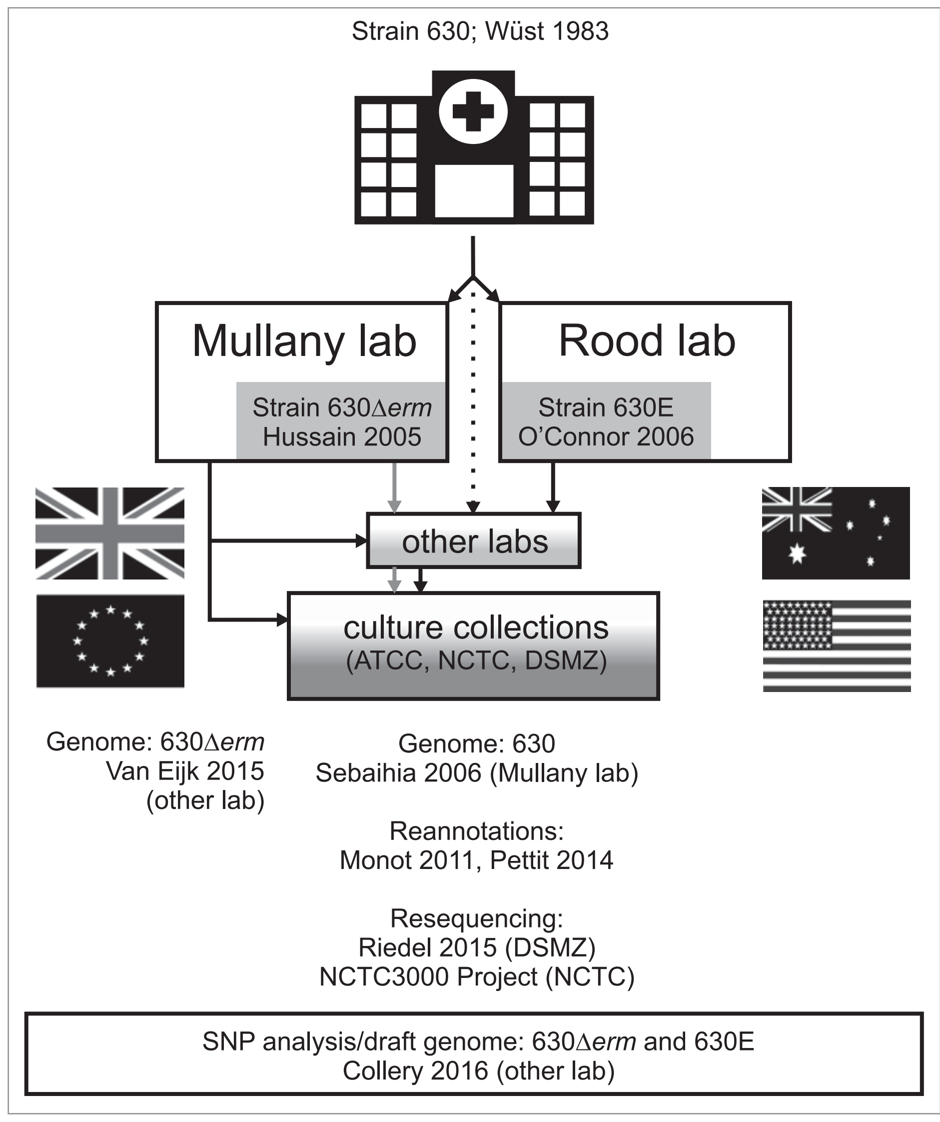

Figure 1. Schematic representation of the genealogy of strain 630, 630Δ*erm* and 630E (JIR8094) and their related genome sequences. Strain 630 was deposited by B. Wren/H. Maschler (ATCC BAA-1382), P. Mullany (NCTC 13307). The DSMZ lists the provenance for 630 (DSMZ 27543) as obtained from P.Bracegirdle (NCTC) and for 630Δ*erm* (DSMZ 28645) as H. Hussain (Mullany laboratory) > N. Minton > R. Gerhard. Figure also highlights that, historically, 630Δ*erm* was the dominant strain used in European *C. difficile* laboratories, whereas 630E (JIR8094) was mainly used in Australia and the USA. The sources of the sequenced DNA are indicated in brackets.

versus 630E). Second, in a comprehensive approach, the authors try to define the contributions of a selected set of single nucleotide polymorphisms (SNPs) to the phenotypic and transcriptomic differences. Instances where a single SNP defines major phenotypic changes are scarce, but not unprecedented; e.g. for *C. difficile*, SNPs in the *gyrA* gene that result in fluoroquinolone resistance underlie the expansion of the epidemic 027/BI/NAP1 strain[35] and for *Campylobacter jejuni* hypervirulence has been linked to SNPs in the outer membrane protein PorA.[36] Also for non-pathogenic model bacteria SNPs can be linked to specific phenotypic changes, related to domestication[37] or adaptation,[38] for instance.

However, considering the number of SNPs, it should not come as a surprise that the authors failed to link SNPs to specific phenotypic differences. Both derivatives differ significantly from strain 630, and though one can argue that 630Δ*erm* more closely resembles the ancestral strain,[24] it is an illusion to consider the findings obtained with this strain to be directly representative for strain 630. These findings are also consistent with other studies that observe substantial phenotypic variation with a specific type of *C. difficile* with respect to, for instance, sporulation.[39-41]

Should we then move away from laboratory strains and research only clinical isolates? While this may address the fact that some regulatory interactions are only observed in certain clinical strains,[42,43] other major issues, such as passaging in laboratories, remain problematic. Moreover, it will further increase the inter-laboratory variation, as each laboratory would have its own "wild type." There is therefore value in the use of a standard strain, and the authors argue that this could be 630Δ*erm*.[24]

In what way could some of the challenges in linking SNPs and phenotypes be addressed? One strategy is to expand the number of strains analyzed to allow for genome wide association studies (GWAS); such studies have demonstrated for instance the relation between SNPs and β-lactam resistance in *Streptococcus pneumonia*[44] and predicted virulence in MRSA from genome data.[45] With a limited set of 14 genome sequences it has already been possible to identify SNPs associated with the epidemic group BI/NAP1/027 that can cause severe disease.[46] With a broader analysis of phenotypic and clinical characteristics and an increasing number of *C. difficile* genome sequences available (on Oct 11, 2016 the number of genome assemblies in Genbank was 647; https://www.ncbi.nlm.nih.gov/genome/?term=clostridioides+difficile), GWAS analysis could contribute significantly to our understanding of this important pathogen.

In summary, the work on *C. difficile* genomes and strains by Collery and coworkers,[24] as well as several others,[39,42,43] should be a caveat to many researchers; their findings may apply only to their specific isolate or strain and should encourage them to be careful with generalizations. Also, researchers should exercise caution in repeated propagation of strains under laboratory conditions and document the provenance even when strains are obtained from reputable sources.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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