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High metabolic versatility of different toxigenic and non-toxigenic *Clostridioides difficile* isolates

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A B S T R A C T

Clostridioides difficile (formerly *Clostridium difficile*) is a major nosocomial pathogen with an increasing number of community-acquired infections causing symptoms from mild diarrhea to life-threatening colitis. The pathogenicity of *C. difficile* is considered to be mainly associated with the production of genome-encoded toxins A and B. In addition, some strains also encode and express the binary toxin CDT. However; a large number of non-toxigenic *C. difficile* strains have been isolated from the human gut and the environment.

In this study, we characterized the growth behavior, motility and fermentation product formation of 17 different *C. difficile* isolates comprising five different major genomic clades and five different toxin inventories in relation to the *C. difficile* model strains 630Δ*erm* and R20291. Within 33 determined fermentation products, we identified two yet undescribed products (5-methylhexanoate and 4-(methylthio)-butanoate) of *C. difficile*. Our data revealed major differences in the fermentation products obtained after growth in a medium containing casamino acids and glucose as carbon and energy source. While the metabolism of branched chain amino acids remained comparable in all isolates, the aromatic amino acid uptake and metabolism and the central carbon metabolism-associated fermentation pathways varied strongly between the isolates. The patterns obtained followed neither the classification of the clades nor the ribotyping patterns nor the toxin distribution. As the toxin formation is strongly connected to the metabolism, our data allow an improved differentiation of *C. difficile* strains. The observed metabolic flexibility provides the optimal basis for the adaption in the course of infection and to changing conditions in different environments including the human gut.

1. Introduction

Clostridioides difficile (previously *Clostridium difficile* (Lawson et al., 2016; Oren and Garrity, 2016)) is considered a major nosocomial pathogen with an increasing number of community-acquired infections (Knight et al., 2015). *C. difficile* infection (CDI) ranges from mild diarrhea to pseudomembranous colitis or sepsis with high morbidity and mortality and have become an extreme economic burden (Nanwa et al., 2015). The transmission of the pathogen is accomplished by the formation of highly resistant spores, which can survive various stress conditions and persist in the environment for months or even up to years (Barra-Carrasco and Paredes-Sabja, 2014). Consequently, *C.*

Abbreviations: BHI, brain heart infusion; CDI, *C. difficile* infection; CoA, Coenzyme A; CFU, colony forming units; CdtLoc, binary toxin locus; CDMM, *Clostridium difficile* minimal medium; OD, optical density; PaLoc, pathogenicity locus

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difficile is a global health threat and can be isolated from mammals, various birds and reptiles, as well as from the environment and food (Hensgens et al., 2012). To current knowledge, symptoms of CDI patients are caused by the production of toxins A (TcdA) and B (TcdB) which lead to extensive intestinal damage and pathology (Carter et al., 2010). The role of the binary toxin CDT is not well understood. Recent data suggest an impact of CDT in host inflammation and host eosinophilic response during infection (Cowardin et al., 2016). To date, PCR ribotyping is an established method for the classification of *C. difficile* strains (Collins et al., 2015).

Currently, the population structure of *C. difficile* comprises six major genomic clades (clades 1–5 and clade C-I) (Dingle et al., 2014). Most of the *C. difficile* isolates were found to belong to the phylogenetic clade 1 containing toxigenic as well as non-toxigenic strains (Dingle et al., 2014). The toxigenic strains of clade 1 feature the presence of a pathogenicity locus (PaLoc) encoding the toxin genes *tcdA* (toxin A) and *tcdB* (toxin B) (Braun et al., 1996; Elliott et al., 2016). Clade 2 and clade 3 isolates include *C. difficile* strains encoding the PaLoc as well as the binary toxin locus (CdtLoc) encoding genes for the subunits CdtA and CdtB together with its response regulator CdtR (Carter et al., 2012; Elliott et al., 2016). The phylogenetic clade 2 encompasses the hypervirulent BI/NAP1/027 isolate, which contains mutations in the *tcdC* gene that is postulated to increase the toxin production (Carter et al., 2011). The phylogenetic clades 4 and 5 were shown to contain toxigenic as well as non-toxigenic *C. difficile* isolates (Dingle et al., 2014; Elliott et al., 2016). Whereas the toxigenic isolates of clade 4 encode only PaLoc-associated genes, *C. difficile* strains belonging to clade 5 can also contain the CdtLoc (Dingle et al., 2014; Elliott et al., 2016). The phylogenetically different clade C-I is currently composed solely of non-toxigenic strains (Dingle et al., 2014). Due to the phylogenetic distance to other clades, the clade C-I may represent a new subspecies within *C. difficile* (Dingle et al., 2014). Most recently, MLST-based studies of *C. difficile* strains revealed two additional clades C-II and C-III (Elliott et al., 2016; Janezic et al., 2016).

Similar to other anaerobic bacteria, *C. difficile* has developed specific pathways to degrade amino acids and sugars by fermentation processes (Elsden and Hilton 1978; Elsden et al., 1976; Mead 1971). Their importance in the human gut is underlined as peptide-fermenting bacteria reach 10^{11} colony forming units per gram dry weight faeces with clostridia and Gram-positive cocci as predominant species (Smith and Macfarlane, 1998). Substrate availability and pH strongly effect the presence of fermentation products directly originating from amino acids or from the central carbon metabolism (Smith and Macfarlane, 1998; Macfarlane and Macfarlane, 2011). During fermentation in *C. difficile*, energy is conserved mainly by substrate-level phosphorylation and the proton-translocating ferredoxin:NAD oxidoreductase RNF complex (Aboulnaga et al., 2013; Buckel and Thauer, 2013). The latter catalyses a process called flavin-based electron bifurcation which can be regarded as a third mode of energy conservation beside substrate level and electron transport phosphorylation (Buckel and Thauer, 2013). In addition to central carbon metabolism-associated fermentation, *C. difficile* shows a highly specific fermentation of amino acids by so-called Stickland reactions which involve the coupled oxidation and reduction of amino acids to short chain organic acids via coenzyme A (CoA)-bound intermediates (Stickland, 1934). Specific amino acids can act as Stickland electron acceptors, Stickland electron donors, or as both leading to a highly specific fermentation profile compared to other clostridia (Elsden et al., 1976; Kim et al., 2006). Instead of the classical Stickland pathway, some amino acids are known to follow modified Stickland reactions e.g. proline and glycine (Cone et al., 1976; Jackson et al., 2006; Seto and Stadtman, 1976; Turner and Stadtman, 1973). Early studies with *C. difficile* revealed its ability to produce Stickland products e.g. from leucine, isoleucine, valine, phenylalanine and tyrosine (Elsden and Hilton 1978; Elsden et al., 1976). Moreover, the oxidative Stickland product of tyrosine, 4-hydroxyphenylacetate, is further processed to the antibiotic substance *p*-cresol by enzymes encoded by the *hpdBCA* operon (locus tags CDIF630erm_00272-00274, Dawson et al., 2011; Dannheim et al., 2017a). The reductive Stickland reactions of phenylalanine as substrate are catalyzed by the same set of enzymes as for leucine (*hadABC*, *ldhA*, *acdB)* with only one single copy for each gene annotated in the model strain 630Δ*erm* (CDIF630erm_00522-00529, Dannheim et al., 2017a). This substrate specificity was previously shown for purified enzymes of *C. difficile* strain DSM 1296^{T} (Kim et al., 2006). In contrast, the oxidative Stickland reactions are catalyzed by two different enzyme sets in *C. difficile*: For branched chain amino acids several copies of the *vor*-operons are annotated in the genome of the model strain 630Δ*erm* (CDIF630erm_00230-00233, 02429-02432, 02669-02672, Dannheim et al., 2017a) while only a single *ior*-operon is annotated for aromatic amino acids (CDIF630erm_02620-02622, Dannheim et al., 2017a). Proteins encoded by the *vor*- and *ior*-operons were shown to have broad substrate specificities as tested in different archaeal organisms (Heider et al., 1996; Mai and Adams, 1994; Ozawa et al., 2012; Tersteegen et al., 1997).

Here, we present an analysis of 33 different fermentation products of 17 *C. difficile* isolates grown to stationary phase in comparison to the two *C. difficile* model strains 630Δ*erm* and R20291. The strains were chosen to cover different clades in combination with a broad variety of different toxin gene inventories. While some central fermentation products showed a similar concentration in all cultures, especially oxidative Stickland products of aromatic amino acids, alcohols and *p*-cresol varied strongly between the different isolates. Our data clearly show the high variety of *C. difficile* fermentation metabolism especially in virulence-connected pathways and adds a new level of classification of isolates beyond genomic and toxin level.

2. Materials and methods

2.1. Strains and cultivation conditions

Strains used in this study are listed in Table 1 and were cultivated from stool samples of infected CDI patients on CLO agar (bioMérieux, Marcy-l'Étoile, France) anaerobically for 48 h at 37°C. Strains were verified and identified using MALDI-TOF mass spectrometry (Bruker

Table 1
Clostridioides difficile strains used in this study. Strains originate from Ghana (GHA), Indonesia (IDN), United Kingdom (UK), Switzerland (CH) or Germany (D).

DSM number	Isolate name	Origin	Ribotype	Clade	Reference
DSM 28666	EC001-01-01	GHA	084	1	Janssen et al. (2016)
DSM 29637	MC012-01-01	IDN	032	1	This work
DSM 29688	SC052-01-01	D	010	1	This work
DSM 29745	SC009-01-01	D	001/72	1	This work
DSM 29632	MC008-01-01	IDN	SLO 160	1	This work
DSM 28668	EC002-01-01	GHA	SLO 228	1	Janssen et al. (2016)
DSM 28196	SC037-01-01	D	027	2	This work
DSM 102978	–	D	nd*	3	Ulrich Nübel
DSM 102859	–	D	023	3	Lutz von Müller
DSM 102860	–	D	127	3	Lutz von Müller
DSM 28669	EC003-01-01	GHA	SLO 091	4	Janssen et al. (2016)
DSM 29629	MC005-01-01	IDN	SLO 235	4	This work
DSM 29627	MC003-01-01	IDN	017	4	This work
DSM 28670	EC004-01-01	GHA	SLO 237	4	Janssen et al. (2016)
DSM 29747	SC052-02-01	D	078	5	This work
DSM 29020	MC002-01-02	IDN	126	5	This work
DSM 101085	3022	D	nd	5	This work
DSM 27147	R20291	UK	027	2	Stabler et al. (2009)
DSM 28645	630Δ*erm*	CH/UK	012	1	Hussain et al. (2005)

* nd, not determined or so far unknown described ribotype.

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