Clostridium difficile glutamate dehydrogenase is a secreted enzyme that confers resistance to H₂O₂

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Clostridium difficile produces an NAD-specific glutamate dehydrogenase (GDH), which converts L-glutamate into α -ketoglutarate through an irreversible reaction. The enzyme GDH is detected in the stool samples of patients with *C. difficile*-associated disease and serves as one of the diagnostic tools to detect *C. difficile* infection (CDI). We demonstrate here that supernatant fluids of *C. difficile* cultures contain GDH. To understand the role of GDH in the physiology of *C. difficile*, an isogenic insertional mutant of *gluD* was created in strain JIR8094. The mutant failed to produce and secrete GDH as shown by Western blot analysis. Various phenotypic assays were performed to understand the importance of GDH in *C. difficile* physiology. In TY (tryptose yeast extract) medium, the *gluD* mutant grew slower than the parent strain. Complementation of the *gluD* mutant with the functional *gluD* gene reversed the growth defect in TY medium. The presence of extracellular GDH may have a functional role in the pathogenesis of CDI. In support of this assumption we found higher sensitivity to H₂O₂ in the *gluD* mutant as compared to the parent strain. Complementation of the *gluD* mutant with the functional *gluD* gene reversed the H₂O₂ sensitivity.

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INTRODUCTION

Clostridium difficile is the leading cause of hospital-acquired diarrhoea, which ranges in severity from mild diarrhoea to fulminant colitis. Antibiotic use is the primary risk factor for the development of *C. difficile* infection (CDI) because it disrupts the normal protective gut flora and enables *C. difficile* to colonize the colon (Bartlett *et al.*, 1978). CDI is estimated to afflict >1 % of all hospitalized patients with a mortality rate approaching 25 % in the elderly and costs the US health care system US\$1–3 billion annually (McGlone *et al.*, 2012; O'Brien *et al.*, 2007). Concurrent with *C. difficile* outbreaks in North America and Europe, the incidence and severity of CDI have increased dramatically over the last decade. The toxins A (TcdA) and B (TcdB) are the major virulence factors that contribute to the pathogenesis of *C. difficile* (Burdon *et al.*, 1981; Kuehne *et al.*, 2010; Lyras *et al.*, 2009), and current diagnosis of CDI is based primarily on the detection of toxins A and B (McCullum & Rodriguez, 2012). One of the earliest tests used for *C. difficile* diagnosis, the latex agglutination test, claimed to detect *C. difficile* toxins, but was later proved to actually detect glutamate dehydrogenase (GDH) (Lyerly *et al.*, 1991). Enzyme immunoassays for the detection of *C. difficile* GDH have been available commercially for the past few years and its detection is

currently performed as part of a two-step algorithm for the diagnosis. An ELISA for *C. difficile* GDH is performed first to confirm the presence of the pathogen and the positive specimens are tested further by toxin ELISA (Carroll, 2011; Shetty *et al.*, 2011). Numerous studies have demonstrated the effectiveness of GDH as a diagnostic marker (Shetty *et al.*, 2011); however, no information is available on the importance of this enzyme for *C. difficile* physiology or pathogenesis.

GDHs are a broadly distributed group of enzymes (Barker, 1981; Merrick & Edwards, 1995) that catalyse the oxidative deamination of glutamate to α -ketoglutarate and ammonia (glutamate + NAD⁺ + H₂O → α -ketoglutarate + NADH + H⁺ + NH₄⁺) or in a reverse reaction they can also catalyse condensation of ammonia and α -ketoglutarate to glutamate with the concomitant oxidation. The physiological role of GDH as either an anabolic or catabolic enzyme is determined by its cofactor (NAD or NADH, NADP or NADPH) specificity. Some GDH enzymes have the ability to act on both directions of the reaction depending on the substrate availability. In *C. difficile*, the GDH enzyme is NAD specific, and mediates the oxidative deamination of glutamate to produce α -ketoglutarate and ammonia (Anderson *et al.*, 1993). *C. difficile* GDH (421 residues, 46 kDa), encoded by *gluD*, is highly homologous (85 % identity) to the GDH of group I *Clostridium botulinum*. Cell-free extracts of proteolytic strains of *C. botulinum* (group I) were found to have high NAD⁺-dependent L-GDH (NAD-GDH) activities compared with non-proteolytic strains (group II) (Hammer & Johnson, 1988). In

Abbreviations: CDI, *Clostridium difficile* infection; GDH, glutamate dehydrogenase; ROS, reactive oxygen species.

Three supplementary figures are available with the online version of this paper.

this study, we detected enzymically active GDH in the supernatants of *C. difficile* cultures.

To understand the importance of this extracellular GDH, we introduced a mutation in the *gluD* gene using the ClosTron technique in *C. difficile* and compared the *gluD* mutant phenotype with the parent strain. Our results suggest that GDH is important for the normal growth of *C. difficile* and the presence of enzymically active extracellular GDH may offer protection to *C. difficile* against H₂O₂ that is released as part of the host defence against CDI.

METHODS

Bacterial strains and growth conditions. *C. difficile* strains R20291 (Stabler *et al.*, 2009), CD646 (Keel *et al.*, 2007), JIR8094 (O'Connor *et al.*, 2006) and the *gluD* mutants (Table 1) were grown anaerobically (10% H₂, 10% CO₂ and 80% N₂) in TY (tryptose yeast extract) broth or TY agar as described previously (Dupuy & Sonenshein, 1998). *Escherichia coli* strain S17-1 (Teng *et al.*, 1998) used for conjugation was cultured aerobically in LB and supplemented with chloramphenicol (30 µg ml⁻¹) or ampicillin (100 µg ml⁻¹), when needed. All routine cloning and plasmid constructions were carried out using standard procedures.

Construction of a *gluD* mutant. A *gluD* mutant in *C. difficile* strain JIR8094 was constructed using the ClosTron gene knockout system (Heap *et al.*, 2007, 2010). The group II intron insertion site in the antisense orientation between nt 324 and 325 of the *gluD* ORF was selected to design intron-retargeting primers, IBS, EBS1d and EBS2 (EBS and IBS denote exon- and intron-binding site, respectively; Table 2). Plasmid retargeting using pMTL007-CE5 was carried out as described previously (Heap *et al.*, 2007, 2010). The resulting plasmid, pMTL007-CE5::Cdi-*gluD*-324a, was transferred to *C. difficile* by conjugation as described previously (Govind & Dupuy, 2012). Insertion of the intron within the target region (*gluD*) in the chromosome confers erythromycin resistance in the resulting mutant strain. Hence, thiamphenicol-resistant transconjugants were resuspended in 200 µl TY broth and plated on TY agar plates containing erythromycin (5 µg ml⁻¹) to select potential L1.LtrB insertions. Putative *gluD* mutants were screened by PCR using *gluD*-specific primers (ORG56 and ORG57) in combination with the EBS-U universal and ERM primers (Table 2). Southern blot analysis was performed as described

previously to verify a specific single integration of the group II intron into the genome (Sirigi Reddy *et al.*, 2013). Genomic DNA (10 µg) was digested with *EcoRV* and separated on a 0.8% agarose gel by electrophoresis. DNA transferred onto Immobilon-Ny+ nylon membranes was prehybridized for 2 h at 60 °C in 5 × SCC/5 × Denhardt's with salmon sperm DNA (100 mg ml⁻¹). The group II intron-specific *ermB* gene and *gluD* gene probes were radiolabelled ([³²P]dATP) using the High Prime kit (Roche) and hybridized overnight in 10 ml fresh pre-hybridization buffer at 60 °C. An end-labelled 1 kb ladder was used as a marker in the blot. The hybridized membrane was washed twice in 2 × SCC/0.5% SDS, and analysed using a phosphor image screen and a Typhoon 9410 scanner (GE Healthcare).

Complementation of the *C. difficile* *gluD* mutant. The *gluD* ORF upstream region (840 bp) along with its ribosome-binding site was PCR amplified from JIR8094 chromosomal DNA using primers *gluDP*(F) and *gluDP*(R) (Table 2), which carried restriction sites *Hind*III and *Xba*I, respectively. The resulted PCR product was digested with *Hind*III and *Xba*I, and was cloned into the *C. difficile* shuttle plasmid pMTL84151 (Heap *et al.*, 2009), digested with the same to yield pRG51 (Table 1). The *gluD* ORF was PCR amplified from JIR8094 chromosomal DNA using primers ORG72 (with *Kpn*I) and ORG79 (with *Sac*I); the PCR product was digested with *Kpn*I and *Sac*I, and cloned into pRG51 to construct plasmid pRG58, where the *gluD* was expressed from its native promoter. Codons for six His residues were introduced in primer ORG79 to express GDH with a C-terminal His-tag. The GDH-expressing plasmid pRG58 and the vector pMTL84151 were introduced into JIR8094 and *gluD* mutant *C. difficile* strains by conjugation. Transconjugants carrying pRG58 or the vector pMTL84151 were grown overnight in TY medium supplemented with thiamphenicol. Aliquots of 10 ml fresh cultures were inoculated with 100 µl overnight cultures and grown for 12 h in TY medium with thiamphenicol. Bacterial cells and the culture supernatants were harvested for the detection of GDH.

***C. difficile* GDH ELISA.** Bacterial cultures were inoculated in 10 ml TY medium and incubated at 37 °C overnight under anaerobic conditions. Culture supernatants were collected and filtered, and the cell pellets were resuspended in 10 mM Tris buffer, pH 8.0 containing a protease inhibitor cocktail (Roche). The cytosolic contents were obtained by sonication of the cells, followed by brief centrifugation to remove unbroken cells and cell debris. Total protein concentration was determined using the Bradford method. Equal amounts of cytosolic fractions (10 µg) and 200 µl culture supernatants collected

Table 1. Bacterial strains and plasmids

Strain/plasmid	Description	Source/reference
<i>Clostridium difficile</i> R20291	NAP1/027 ribotype, isolated in 2006 following an outbreak in Stoke Mandeville Hospital, UK	Stabler <i>et al.</i> (2009)
<i>Clostridium difficile</i> CD646	078 ribotype (swine isolate)	Keel <i>et al.</i> (2007)
<i>Clostridium difficile</i> JIR8094	630 erythromycin-sensitive derivative (012 ribotype)	O'Connor <i>et al.</i> (2006)
<i>Clostridium difficile</i> JIR8094 <i>gluD</i> ::CT	JIR8094 with intron insertion within the <i>gluD</i> gene	This study
<i>Escherichia coli</i> DH5α	<i>endA1 recA1 deoR hsdR17</i> (r _K ⁻ m _K ⁺)	NEB
<i>Escherichia coli</i> S17-1	Strain with integrated RP4 conjugation transfer function; favours conjugation between <i>E. coli</i> and <i>C. difficile</i>	Teng <i>et al.</i> (1998)
pMTL007-CE5	ClosTron plasmid	Heap <i>et al.</i> (2007, 2010)
pMTL007-CE5::Cdi- <i>gluD</i> -324a	pMTL007-CE5 carrying <i>gluD</i> -specific intron	This study
pMTL84151	<i>E. coli</i> / <i>C. difficile</i> shuttle plasmid	Heap <i>et al.</i> (2009)
pRG51	pMTL84151 containing <i>gluD</i> promoter	This study
pRG58	<i>gluD</i> with 6His in pRG51	This study

Table 2. Oligonucleotides

Name	Direction	Sequence (5'→3')
gluDP (F)	Forward	AAGCTTcaaggttttagctgggatatcgg
gluDP (R)	Reverse	TCTAGATGACATttgaaagcccttataaata
gluD-323 324a	IBS	AAAAAAGCTTATAATTATCCTTAACTATCATTCCAGTGCGCCCAGATAGGGTG
gluD-323 324b	EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATTCCACCTAACTTACCTTTCTTTGT
gluD-323 324a	EBS2	TGAACGCAAGTTTCTAATTTTCGATTATAGTTTCGATAGAGGAAAGTGTCT
EBS-U		CGAAATTAGAACTTGCCTTCAGTAAAC
ERM		GGTCGGTACCATGGACCCAAGAGATGCTGGTGCTTCT
ORG72	Forward	GGTACCATGTCAGGAAAAGATGTAAATGTCCTTCGAG
ORG73	Forward	GGTACCATGGAACCAAGCAGTTTATGAATTATTAATAA
ORG79	Reverse	GAGCTCTTAATGATGATGATGATGATGGTACCATCCTCTTAATTTTCATAGC
ORG56	Forward	ATGTCAGGAAAAGATGTAAATGTCCTTCGAGATGG
ORG57	Reverse	TTAGTACCATCCTCTTAATTTTCATAGCTTCAGCAA

at different time points of bacterial growth were assayed for GDH protein using the *C. difficile* CHEK-60 GDH (TECHLAB) ELISA kit following the manufacturer's directions.

Western blot analysis. To detect GDH, the cytoplasmic proteins harvested from the bacterial cultures were separated by SDS-PAGE and immobilized onto PVDF membranes employing a semidry blot technique. Membranes were probed subsequently with antibodies specific for GDH (1:200) which is present in the enzyme conjugate supplied with the CHEK-60 GDH ELISA kit. The membrane with test samples was probed with antibodies against ribosomal proteins L7/L12 (Kolberg *et al.*, 1997) at a dilution of 1:5000, when necessary. Dot-blot assays were performed with concentrated culture supernatants and cytoplasmic proteins to detect ribosomal proteins and GDH. Proteins of specific concentrations were spotted on PVDF membranes and the membranes were processed as described above.

GDH activity staining. GDH in-gel activity was detected following the protocol described previously (Okwumabua *et al.*, 2001). The mutant and the parent strains were grown for 16 h in 100 ml TY medium with or without erythromycin (5 µg ml⁻¹). Culture supernatants and cytosolic extracts were obtained as described above. The culture supernatants were concentrated 10-fold using the Amicon 8000 series stirred cell fitted with an ultrafiltration membrane with a molecular mass cut-off range of 10 kDa. The concentrated supernatant and cytosolic extracts were subjected to non-denaturing PAGE. Briefly, the samples were resuspended in sample buffer devoid of any denaturing agents and were separated in non-SDS-polyacrylamide gels where acrylamide concentrations of 3 % and 7 % (w/v) were used in the stacking and resolving gels, respectively. Electrophoresis was performed in Tris-glycine buffer without SDS at 50 V. Proteins with NAD-specific GDH activity were visualized by immersing the gels in 20 ml of 20 mM Tris/HCl (pH 8.0) reaction buffer with nitro blue tetrazolium (0.3 mg ml⁻¹) and phenazine methosulfate (0.05 mg ml⁻¹). To detect NAD- or NADH-specific GDH activity, either L-glutamate with 0.5 mM NAD or 50 mM α-ketoglutarate with 1 mM NADH was added to the reaction buffer, respectively. GDH activity could be detected as purple-coloured bands in the gels.

Growth curve and H₂O₂ sensitivity assay. Bacterial susceptibility to H₂O₂ was tested by the radial diffusion assay. An aliquot of 1 ml of exponentially growing bacterial cultures (8 h old) was mixed with 2 ml TY agar (0.8 %) and the mixture was poured onto TY agar plates; filter paper disks (diameter 6 mm) impregnated with 15 µl H₂O₂ (1 %) or 200 mM menadione were placed on the agar surface. After overnight incubation at 37 °C, the inhibition growth zone was measured (in millimetres). Each experiment was performed three

times to confirm reproducibility. Mean values are plotted with error bars representing the SD for each strain and condition.

The growth patterns of parent and *gluD* mutant strains were studied in TY medium using a Bioscreen C plate reader that was kept inside the anaerobic chamber. Overnight cultures were first diluted 10-fold and 15 µl of these diluted cultures was used to inoculate 150 µl TY medium in each well. The plate temperature was maintained at 37 °C throughout the growth and the OD₆₀₀ was measured every 30 min after 10 s shaking of the plate. To measure the bacterial growth in the presence of H₂O₂, bacterial strains were grown in TY medium with 50 µM H₂O₂ and the OD₆₀₀ was measured at regular intervals using a Bioscreen C plate reader.

Bacterial survival upon exposure to H₂O₂. Bacterial cultures (10 ml) were grown for 6 h in TY medium before splitting them into two 5 ml cultures. To one 5 ml aliquot, H₂O₂ was added to a final concentration of 100 µM and this was incubated for 5 min. After this short exposure, medium with H₂O₂ was removed by centrifugation and the bacterial pellet was washed with sterile PBS twice before resuspending with 1 ml TY medium. Serial dilutions were made to measure the number of bacteria that survived H₂O₂ exposure. The unexposed 5 ml aliquot was processed similarly and was used to estimate the total number of bacteria used for the treatment. The rate of cell survival was calculated as the number of viable cells after exposure to H₂O₂ divided by the total number of viable cells before treatment.

RESULTS

GDH is secreted into *C. difficile* culture supernatants

We first observed extracellular GDH in *C. difficile* cultures whilst investigating the mechanism of toxin secretion (Govind & Dupuy, 2012). This was the initial evidence that GDH is secreted from *C. difficile* and can be detected in stool samples. To confirm this observation, we further collected culture supernatants from three different *C. difficile* strains at exponential growth stage (6 h) and tested for the presence of GDH using ELISA. Cytosolic proteins collected from the same bacterial strains were used as positive controls. GDH was detected in the culture supernatants of all three *C. difficile* strains tested (Fig.

1a). We then tested the culture supernatants and the cytosolic proteins collected at different time points during growth of strain JIR8094. Culture supernatants collected at different time points were equalized based on the OD₆₀₀ recorded at that time point before GDH ELISA analysis. Our results showed that cytosolic GDH levels stayed constant throughout growth and we could detect GDH in the culture supernatants even in 4 h cultures. As the bacterial culture entered the stationary phase, more GDH accumulated in the culture supernatant (Fig. 1b). To check whether extracellular GDH is the result of cell lysis, we looked for the cytosolic ribosomal L7/L12 proteins in the culture supernatants collected at early exponential growth (6 h). Equal amounts of concentrated supernatant and

cytosolic proteins were spotted on a PVDF membrane and probed with mAbs against L7/L12 ribosomal proteins. The results (Fig. 1c) showed that only GDH and not the ribosomal proteins L7/L12 could be detected in the culture supernatants, indicating that GDH enzyme release is not due to cell lysis.

To determine whether the extracellular GDH is enzymically active, we collected the culture supernatants from JIR8094 strain at 6 h and assayed for GDH enzyme activity by zymogram. The concentrated culture supernatant proteins along with the cytosolic proteins were separated by non-denaturing PAGE, and were processed using glutamate and NAD as substrates to detect GDH enzymic activity (Fig.

Fig. 1. Presence of enzymically active extracellular GDH in *C. difficile* cultures. (a) Detection of GDH in the culture supernatant and cytoplasmic fractions harvested from different *C. difficile* strains using ELISA (CHEK-60 GDH). Proteins were harvested from 6 h cultures. The data shown are the mean $\pm$ SD of three replicate samples. (b) Detection of GDH in samples harvested at different time intervals from JIR8094 bacterial culture using ELISA (representative experiment of three independent assays). The amount of GDH in the sample is directly proportional to the absorbance recorded at 405 nm. (c) Cytoplasmic and culture supernatant proteins harvested from the JIR8094 strain were analysed in dot-blots using antibodies against GDH and L7/L12 ribosomal proteins. (d, e) Zymogram to detect GDH enzyme activity in JIR8094 culture supernatant and cytoplasmic protein samples. (d) NAD-specific GDH activity was tested using NAD and glutamate as substrate. (e) NADH-specific GDH activity was tested using NADH and α -ketoglutarate as substrate.

1d). A single band of ~150 kDa was detected both in the cytosolic extracts and in the culture supernatants, indicating the enzymically active enzyme to be a homotrimer. Similar homotrimeric GDH has been observed in *Thermus thermophilus* (Bolivar *et al.*, 2008). No enzyme activity was detected in the samples when α -ketoglutarate and NADH were used as substrates and served as negative controls (Fig. 1e). These results demonstrate that extracellular GDH proteins in *C. difficile* are enzymically active.

Construction and characterization of *C. difficile* *gluD* mutant

To understand the importance of GDH in *C. difficile* physiology, we created insertional mutations in *gluD* in *C.*

difficile JIR8094 strain using the ClosTron technique (Fig. S1a, available in *Microbiology Online*). Intron insertion in the *gluD* gene was confirmed by PCR and Southern blot hybridization (Fig. S1b, c), and the absence of the GDH protein in the mutant was confirmed through Western blotting with anti-GDH antibody (Fig. S1d). The growth rates of the *gluD* mutant and the parent strains were compared. Following delayed entry into exponential growth, the *gluD* mutant grew at a rate approaching that of the parent strain (Fig. S1e). Sporulation efficiency of the *gluD* mutant was found to be equivalent to the WT strain (Fig. S2).

To complement the *gluD* mutant, the plasmid pRG58 was constructed by cloning the WT *gluD* along with a C-terminal 6 His-tag and 800 bp of its own upstream DNA

Fig. 2. Complementation of the JIR8094 *gluD* mutant. (a) Detection of GDH in the complemented strains. Bacterial cultures were grown for 6 h, and the cytoplasmic proteins were harvested and separated by SDS-PAGE, transferred onto PVDF membranes, and probed with anti-GDH antibody. (b) Detection of GDH enzyme activity in the complemented *gluD* mutant strains. Culture supernatants were harvested from 6 h bacterial cultures and separated in non-denaturing gels as described in Methods to detect GDH enzyme activity. Bovine GDH and the cytosolic protein from the complemented strain were used as positive controls. (c) The same samples used in (b) were separated by SDS-PAGE and were probed with anti-L7/L12 ribosomal antibodies. (d) Growth curves of parent and complemented GDH mutant strains. Error bar, SEM from 10 replicates (result from a representative experiment out of three independent experiments).

sequence, which contains the putative *gdh* promoter. The plasmid pRG58 was introduced into the JIR8094 *gluD* mutant by conjugation. Expression of GDH protein in the complemented strain was confirmed by Western blotting with GDH antibody (Fig. 2a). The complemented mutant grew similarly to the parent strain with vector alone (Fig. 2d), confirming that the reduced growth rate in the mutant was due to the lack of GDH activity. We then looked for the presence of extracellular GDH activity in the complemented strain. Culture supernatants from the bacterial cultures were collected at 6 h after inoculation and concentrated 10-fold before testing for GDH activity. Bovine GDH (Sigma Aldrich) and the complemented strain's cytosol were used as a positive control. The results showed that GDH expressed in the complemented strain is active and could be detected in the extracellular fraction (Fig. 2b). The same samples were probed for the presence of ribosomal protein L12/L7 by Western blot. The cytosolic ribosomal protein was detected only in the JIR8094 cytosol and not in culture supernatants tested. This confirmed that the GDH is released in the absence of cell lysis from the parent and the complemented strain (Fig. 2c).

C. difficile gluD mutants are more sensitive to H₂O₂

The *gluD* mutant along with the parent JIR8094 strain was subjected to various phenotypic assays. Initially, growth characteristics of the *C. difficile gluD* mutant in the defined medium with and without glutamate or glutamine were tested. Absence of GDH in the *gluD* mutant did not impair its growth in the absence of glutamate or glutamine in the defined medium (Fig. S3). Further, Biolog phenotype microarrays were employed to identify phenotypes specific for the *gluD* mutant. No significant difference between the parents and the *gluD* mutant could be observed in the phenotypic microarray except for moderate sensitivity of the *gluD* mutant to menadione (data not shown), a reactive oxygen species (ROS)-generating agent. This gave us an initial clue about the *gluD* mutant's sensitivity to ROS. We subsequently analysed the *gluD* mutant and parent strain for their sensitivity to menadione and H₂O₂.

In the radial diffusion assay with 100 mM menadione, a smaller zone of inhibition was observed for the parent strain when compared with the *gluD* mutant, but the difference was not statistically significant ($P>0.05$). Greater sensitivity of the *gluD* mutant to ROS was observed when H₂O₂ was used, where a significantly larger zone of inhibition was seen as compared with the parent strain and with the *gluD* mutant complemented with functional GDH ($P<0.001$) (Fig. 3). Further, we monitored the bacterial growth of the complemented JIR8094 *gluD* mutant in the presence of a sublethal concentration of H₂O₂ (50 μ M). JIR8094 and its *gluD* mutant with vector alone were used as controls in this experiment. In the presence of H₂O₂, there was a delay in the initiation of growth of the parent JIR8094 strain for up to 5 h after inoculation and no

growth was observed for the JIR8094 *gluD* mutant strain with vector (Fig. 4a, b). The *gluD* mutant complemented with GDH (pRG58) grew similarly to the parent strain in the presence of H₂O₂ (Fig. 4c). When exposed to a lethal dose of H₂O₂ for a short time, a higher survival rate was observed for the JIR8094 parent strain compared to its *gluD* mutant (Fig. 4d).

DISCUSSION

GDH ELISA is used routinely as one of the diagnostic tools to detect CDI (Shetty *et al.*, 2011). The presence of GDH in patients' stool samples shows the presence of the organism and implies that the enzyme is produced *in vivo* during CDI. In this study, we report that enzymically active *C. difficile* GDH can be detected in *C. difficile* culture supernatants. Lack of cytoplasmic ribosomal protein L7/L12 in the culture supernatant of a 6 h *C. difficile* culture indicates that GDH is secreted from the cell in the absence of cell lysis. NADH-dependent GDHs play an anabolic role where they catalyse the assimilation of ammonia by reductive amination of α -ketoglutarate to form L-glutamate (Hudson & Daniel, 1993). Many other amino acids are then synthesized from L-glutamate by transamination. In the NAD-dependent GDH enzyme reaction, the enzyme serves in a catabolic function, catalysing the oxidative deamination of L-glutamate to α -ketoglutarate (Hudson & Daniel, 1993). The GDH enzyme from *C. difficile* has been characterized previously to be a NAD-dependent enzyme and is involved in the degradation of glutamate (Anderson *et al.*, 1993). *C. difficile* GDH mutants created in this study grew slower than their respective parent strains. This result

Fig. 3. Radial diffusion assays were conducted by using 1 % H₂O₂ and 100 mM menadione. Histograms express mean ($\pm$ SD; minimum $n=3$) zones of growth inhibition. Student's *t*-test was performed for statistical evaluation.

Fig. 4. H₂O₂ sensitivity of *gluD* mutants. Bacterial strains were grown in the absence or presence of sublethal doses of H₂O₂ and their OD₆₀₀ recorded at regular intervals using a Bioscreen C plate reader. Results of one representative experiment of three independent experiments are shown. Error bar, SEM from 10 replicates. (a) Parent strain with vector alone. (b) *gluD* mutant with vector alone and (c) *gluD* mutant complemented with GDH. (d) Survival of bacterial strains upon exposure to a lethal dose of H₂O₂. Bacterial cells (10⁸ cells ml⁻¹) were incubated with H₂O₂ for 5 min. All values represent mean (± SD; minimum *n*=3) per cent survival compared the unexposed cells (100%; *n*=3) and were compared using Student's *t*-test.

suggests that GDH is an important metabolic enzyme and is necessary for efficient bacterial growth. It is well known that fermentation of amino acids serves as an important source of energy in this anaerobic pathogen (Jackson *et al.*, 2006). Interestingly, in the epidemic *C. difficile* strain R20291, despite repeated attempts, we could not introduce an insertional mutation into *gluD*. The reasons for this are unclear, but construction of mutants in R20291 is more difficult than in JIR8094, suggesting this might be a technical issue. The R20291 strain was isolated during an outbreak in the UK and belongs to ribotype 027/NAP01. This strain carries mutations in *tcdC* and its genome sequence revealed the presence of 234 additional genes when compared with strain 630 (Stabler *et al.*, 2009). Thus, in the genetic background of R20291, the *gluD* gene may be essential for growth. The use of alternative mutagenesis strategies may help resolve this question (Faulds-Pain & Wren, 2013; Ng *et al.*, 2013).

Typically, bacterial GDHs are cytoplasmic or cytoplasmic membrane-associated proteins. In *C. difficile*, GDH could be detected both in the cytoplasm and in extracellular culture supernatants. Preliminary experiments from our lab suggest that secretion is independent of the N-terminal signal sequence (data not shown). Further investigation is underway to understand the secretion mechanism of GDH from *C. difficile*. Extracytoplasmic NAD-dependent GDH has been reported in *Streptococcus suis* and *Porphyromonas gingivalis* (Joe *et al.*, 1994; Okwumabua *et al.*, 2001). Many other normally intracellular enzymes have been reported to be present outside the cell in some bacteria. For example, glyceraldehyde 3-phosphate dehydrogenase was found to be surface-associated in streptococci (Lottenberg *et al.*, 1992) and in the invasive parasite *Schistosoma mansoni* (Goudot-Crozel *et al.*, 1989). In streptococci, the surface-associated glyceraldehyde 3-phosphate dehydrogenase enzyme appears to bind to a variety of host proteins, and was

found to be important for bacterial colonization and invasion in the host (Pancholi & Fischetti, 1992; Terao *et al.*, 2006). The role of extracellular GDH in *C. difficile* remains open for speculation. Since the extracellular GDH is enzymically active, it could be used by the bacteria for the acquisition and utilization of free glutamate in the host gut. NAD and the glutamate released from the lysed host cells could be utilized by *C. difficile* GDH to generate NADH, but it is highly unlikely that the bacteria would use this extracellular NADH to generate energy. Hence, one possible function for extracellular NADH would be to generate a reducing environment for the efficient growth of strictly anaerobic *C. difficile*. Other anaerobic pathogens *P. gingivalis* and *C. botulinum* may also utilize their extracytoplasmic GDH for a similar purpose.

Glutamate in the intestinal tract has been shown to function as a signalling molecule in the enteric nervous system by binding to specific receptors to regulate many gut functions (Kirchgesner, 2001). Glutamate receptors have also been identified in non-neuronal cells such as lymphocytes and were found to influence their function to modulate immune responses (Xue & Field, 2011). By scavenging glutamate, an important signalling molecule, *C. difficile* may affect efficiently many host functions, including the immune response.

Interestingly, in this study we found the *C. difficile* GDH mutants to be more sensitive to H₂O₂ than the parent strains. The reasons for this H₂O₂ sensitivity in GDH mutants are not clear. One possibility is that the absence of an important energy metabolism enzyme impairs the bacterium's defence against H₂O₂. To evaluate this hypothesis, we tested the sensitivity of GDH mutants for other antibacterial agents. The MICs for other antibacterial agents such as rifampicin, metronidazole and vancomycin were found to be similar for both parent and GDH mutants (data not shown). Recent scientific evidence strongly suggests that α -ketoglutarate, the product of the NAD-GDH enzymic action, is important for managing oxidative stress in both prokaryotes and eukaryotes. When *Pseudomonas fluorescens* was grown in the presence of menadione, a ROS-generating agent, the GDH activity in the cells increased to generate α -ketoglutarate (Mailloux *et al.*, 2009). In the same study, it was shown that α -ketoglutarate could sequester H₂O₂ by non-enzymically decarboxylating itself into succinate (Mailloux *et al.*, 2009). α -Ketoglutarate also plays an important role in eukaryotic cells by regulating activity of hypoxic inducible factor-1 α in response to oxidative stress (Semenza, 2007). In plants, hypoxic stress resulted in the production of more α -ketoglutarate by upregulating NAD-dependent GDH activity (Limami *et al.*, 2008). Hence, it is highly likely that α -ketoglutarate generated through the action of GDH in *C. difficile* can contribute to H₂O₂ tolerance. CDIs are marked by a high rate of intestinal inflammation triggered by the host immune response. Producing extracellular GDH during infection may be one strategy by which *C. difficile* defends itself from ROS generated during the host immune response.

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