**Clostridium difficile** glutamate dehydrogenase is a secreted enzyme that confers resistance to H$_2$O$_2$

Brinthra Prasummanna Girinathan, Sterling E. Braun and Revathi Govind

**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$1–3 billion annually (McGlone *et al.*, 1981; Kuehne *et al.*, 2010; Lyras *et al.*, 2011). Concurrent with CDI, an isogenic insertional mutant of *gluD* was created in strain JIR8094. The mutant failed to produce and secrete GDH as shown by Western blot analysis. Various phenotypic assays were performed to understand the importance of GDH in *C. difficile* physiology. In TY (tryptose yeast extract) medium, the *gluD* mutant grew slower than the parent strain. Complementation of the *gluD* mutant with the functional *gluD* gene reversed the growth defect in TY medium. The presence of extracellular GDH may have a functional role in the pathogenesis of CDI. In support of this assumption we found higher sensitivity to H$_2$O$_2$ in the *gluD* mutant as compared to the parent strain. Complementation of the *gluD* mutant with the functional *gluD* gene reversed the H$_2$O$_2$ sensitivity.

GDHs are a broadly distributed group of enzymes (Barker, 1993; Anderson *et al.*, 1993) that catalyse the oxidative deamination of glutamate to α-ketoglutarate and ammonia (glutamate + NAD$^+$ + H$_2$O → α-ketoglutarate + NADH + H$^+$ + NH$_4^+$) 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 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.
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 *gldD* gene using the ClosTron technique in *C. difficile* and compared the *gldD* mutant phenotype with the parent strain. Our results suggest that GDH is important for the normal growth of the mutant phenotype with the parent strain. Our results also indicate that GDH may offer protection to *C. difficile* against H$_2$O$_2$ 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 *gldD* mutants (Table 1) were grown anaerobically (10% H$_2$, 10% CO$_2$ and 80% N$_2$) in TY (tryptose yeast 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 gldD mutant.** A *gldD* 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 *gldD* ORF was PCR amplified from JIR8094 chromosomal DNA using primers ORG72 (with KpnI), ORG73 (with XbaI) and cloned into pRG51 to construct plasmid pRG58, where the *gldD* 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 *gldD* 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 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× SSC/5× Denhardt's with salmon sperm DNA (100 μg ml$^{-1}$). The group II intron-specific *ermB* gene and *gldD* 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× SSC/0.5% SDS, and analysed using a phosphor image screen and a Typhoon 9410 scanner (GE Healthcare).

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium difficile</em> R20291</td>
<td>NAP1/027 ribotype, isolated in 2006 following an outbreak in Stoke Mandeville Hospital, UK</td>
<td>Stabler et al. (2009)</td>
</tr>
<tr>
<td><em>Clostridium difficile</em> CD646</td>
<td>078 ribotype (swine isolate)</td>
<td>Keel et al. (2007)</td>
</tr>
<tr>
<td><em>Clostridium difficile</em> JIR8094</td>
<td>630 erythromycin-sensitive derivative (012 ribotype)</td>
<td>O’Connor et al. (2006)</td>
</tr>
<tr>
<td><em>Clostridium difficile</em> JIR8094 gluD::CT</td>
<td>JIR8094 with intron insertion within the <em>gldD</em> gene endA1 recA1 deoK hsdR17 (rK mC2)</td>
<td>O’Connor et al. (2006)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5x</td>
<td>Strain with integrated RP4 conjugation transfer function; favours conjugation between <em>E. coli</em> and <em>C. difficile</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli</em> S17-1</td>
<td>ClosTron plasmid</td>
<td>Teng et al. (1998)</td>
</tr>
<tr>
<td>pMTL007-CE5</td>
<td>pMTL007-CE5 carrying <em>gldD</em>-specific intron</td>
<td>Heap et al. (2007, 2010)</td>
</tr>
<tr>
<td>pMTL084151</td>
<td><em>E. coli/C. difficile</em> shuttle plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>pRG51</td>
<td>pMTL84151 containing <em>gldD</em> promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pRG58</td>
<td><em>gldD</em> with 6His in pRG51</td>
<td>This study</td>
</tr>
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</table>
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 (Okumubaua 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 bars representing the SD for each strain and condition. 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.}

### Table 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Direction</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gluDP (F)</td>
<td>Forward</td>
<td>AAGCTTcaaggttagctgggtatcgg</td>
</tr>
<tr>
<td>gluDP (R)</td>
<td>Reverse</td>
<td>TCTAGATGACATttttagctgggtatcgg</td>
</tr>
<tr>
<td>gluD-323324a</td>
<td>IBS</td>
<td>AAAAAAGCTTATATTTATCTTGTTACATTCGTCGACGCGATAGGGT</td>
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<tr>
<td>gluD-323324b</td>
<td>EBS1d</td>
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<td>EBS-U</td>
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<td>ERM</td>
<td></td>
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<td>ORG73</td>
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<td>ORG79</td>
<td>Reverse</td>
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<tr>
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<td>Forward</td>
<td>ATGTCAAGGAAAAGATGTTAATGGGTCGAGATG</td>
</tr>
<tr>
<td>ORG57</td>
<td>Reverse</td>
<td>TTAGTACCATCCCTAATTTTTGATTATGAGTGGAGAAAGTGTC</td>
</tr>
</tbody>
</table>

**Oligonucleotides**

- **gluDP (F)**: AAGCTTcaaggttagctgggtatcgg
- **gluDP (R)**: TCTAGATGACATttttagctgggtatcgg
- **gluD-323324a**: AAAAAAGCTTATATTTATCTTGTTACATTCGTCGACGCGATAGGGT
- **gluD-323324b**: CAGATGGTGACATTGTTACATTCGTCGACGCGATAGGGT
- **gluD-323324a**: TGAACGGCAAGTTCCTAATTTTTGATTATGAGTGGAGAAAGTGTC

- **EBS-U**: CGAATTAGAAACTTGGTGTCGATGAAAC
- **ERM**: GGTCTGATCCATGGAACCGAATGATGGTGCCTT
- **ORG72**: GTTACCATGTCAGGAAAGATGTTAATGGGTCGAG
- **ORG73**: GTTACCATGGAACCGAATGTTAATGGGTCGAG
- **ORG79**: GAGCTCTTAATGTAGTGATGATGAGTGATGACCATCCTCTTTCTCAAC
- **ORG56**: ATGTCAAGGAAAAGATGTTAATGGGTCGAGATG
- **ORG57**: TTAGTACCATCCCTAATTTTTGATTATGAGTGGAGAAAGTGTC
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 OD$_{600}$ 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. 1d, e).
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* **

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.
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 μM). 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 (± SD; minimum n=3) zones of growth inhibition. Student’s t-test was performed for statistical evaluation.](image-url)
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

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**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 (10⁶ 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.
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 (Kirchgessner, 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. However, 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 (Malloux et al., 2009). In the same study, it was shown that α-ketoglutarate could sequester H2O2 by non-enzymically decarboxylating itself into succinate (Malloux et al., 2009). α-Ketoglutarate also plays an important role in eukaryotic cells by regulating activity of hypoxic inducible factor-1a 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.

ACKNOWLEDGEMENTS

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