Corynebacterium diphtheriae putative tellurite-resistance protein (CDCE8392_0813) contributes to the intracellular survival in human epithelial cells and lethality of Caenorhabditis elegans

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Tellurium (Te) is a metalloid that exists as a trace component in natural environments. Te compounds are used in industrial processes and increased delivery of these compounds into the environment generates pollution in soil and water, potentially resulting in contamination and subsequent adverse effects on public health (Avazeri et al. 1997, Aradská et al. 2013). Tellurite (TeO₄²⁻), one of the most oxidised and soluble forms of Te, is toxic to both procaryotes and eukaryotes. Among procaryotes, Gram-negative bacteria are particularly susceptible to Te salts, whereas some Gram-positive bacteria are naturally resistant. Te compounds have a long history as antimicrobial and therapeutic agents for the treatment of infectious diseases, e.g., hanseniasis and tuberculosis. Although Te compounds have not been overlooked as antimicrobial agents, recent investigations studies concerning their biochemical properties and toxicity mechanisms of these molecules have identified Te compounds as potential candidates for use as antibiotics, anticancer drugs and therapeutic agents for the treatment of Parkinson’s disease (Ba et al. 2010, Sekhon 2013). Currently, TeO₄²⁻ is used as a selective agent in the culture media of some pathogens, such as Corynebacterium diphtheriae and Staphylococcus aureus (Taylor 1999).

Although little is known about the mechanisms of TeO₄²⁻-resistance (Te⁸), numerous plasmid and/or chromosomally encoded Te⁸ determinants have been identified in different bacterial species, including human pathogens. The presence of Te⁸ determinants in pathogenic bacteria suggests that these genes might provide some selective advantage in the environment and might also be associated with pathogenicity (Pei et al. 2013, Franks et al. 2014). Previous studies have demonstrated that these determinants are involved in resistance to bacteriophages and colicins (Whelan et al. 1993), antiseptics and disinfectants (Teitzel & Parsek 2003) and antimicrobials (Collins et al. 2010, Pei et al. 2013, Franks et al. 2014). In addition, these genes have also been implicated in adherence to epithelial cells (Yin et al. 2009, Pei et al. 2013) and susceptibility to reactive oxygen species (ROS) (Franks et al. 2014).

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Currently, various virulence factors of *C. diphtheriae* have been described, including the production of potent exotoxin, the formation of biofilms (Gomes et al. 2009), adherence, invasion and survival within different types of human cells (Hirata Jr et al. 2002, Bertuccini et al. 2004, Santos et al. 2010, Peixoto et al. 2014). The major aetiologic agent of diphtheria is also one of the most well-known Te⁶ pathogens. However, the mechanisms involved in this resistance and its relevance in the pathogenicity of *C. diphtheriae* remain unknown. Therefore, in the present study, we detected a putative Te⁶ determinant in *C. diphtheriae* strain CDC-E8392 (CDC8392_0813 protein) and showed a role for this gene in *C. diphtheriae* infection, analysing its effects on resistance to antimicrobial agents and hydrogen peroxide (H₂O₂), adherence to biotic and abiotic surfaces, intracellular survival and ability to kill nematodes.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions - *C. diphtheriae* strains** used in this study and their characteristics are listed in Table I. These microorganisms were maintained in trypticase soy broth (TSB) (BD Difco™, USA) at 37°C and stored in the same medium with 20% glycerol. *Escherichia coli* TOP10 Electrocomp™ (Thermo Fisher Scientific Inc Invitrogen™, USA) and *E. coli* OP50 were grown in Luria Bertani (BD Difco™) medium at 37°C. When appropriate, kanamycin (Sigma-Aldrich Co, USA) was added (50 µg mL⁻¹).

*In silico search and three-dimensional model prediction of the putative Te⁶ protein - An in silico search for resistance determinants in *C. diphtheriae* strains was conducted through the Protein Bank of the National Center for Biotechnology Information. A hypothetical protein included in the TeO₂⁻ resistance/dicarboxylate transporter family was identified in all sequenced strains.*

The Phyre2 server was used to predict the three-dimensional structure of the putative Te⁶ protein of *C. diphtheriae* (Kelley & Sternberg 2009, Guo et al. 2012, Torktaz et al. 2012, Nema & Pal 2013). Two parameters were considered to select the best model: confidence and coverage.

**Disruption of the putative Te⁶ gene - For the chromosomal disruption of the *C. diphtheriae* putative Te⁶ gene, the TOPO® TA Cloning® Kit (Thermo Fisher Scientific Inc Invitrogen™) was used. The CDC-E8392 strain was chosen as template and a 207 bp internal DNA fragment from CDC8392_0813 gene was amplified via polymerase chain reaction (PCR) with the following primer pair: 5'-TCGTTTTATGCGGGTGAC-3' and 5'-GGGTGGCCTTGGCACTTGATG-3'. Amplification was performed with an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min and 30 s and a final elongation step at 72°C for 10 min.*

The DNA fragment was ligated to the overhanging 3'-deoxothymidine of linearised pCR2.1-TOPO vector provided in the kit. The resulting plasmid “pCR2.1-TOPO-CDC8392_0813” was propagated in *E. coli* TOP10 Electrocomp™ according to the manufacturer’s instructions and isolated using the PureLink Quick DNA Mini-prep Kit (Thermo Fisher Scientific Inc Invitrogen™). Five
micrograms of unmethylated plasmid isolated from this *E. coli* strain was used to transform *C. diphtheriae* using a GenePulser II (Bio-Rad Laboratories Inc, USA), as previously described (Dorella et al. 2006). The electroporated cells were added to 1 mL of TSB and incubated for 4 h at 37°C. The culture was plated onto a TSB agar plate containing kanamycin and maintained at 37°C for 48 h. Because pCR2.1-TOPO cannot autonomously replicate in *C. diphtheriae*, kanamycin-resistant *C. diphtheriae* carried the vector, integrated via recombination into the chromosomal CDC8392_0813 gene and designated LDCIC-L1.

To confirm the insertion of the plasmid into the chromosome of the *C. diphtheriae* strain CDC-E8392, PCR reactions using primers aligned at the start and stop codons of the CDC8392_0813 gene (5'-TCGTATCATTGGTGCCACAG-3' and 5'-AATCGTGCCGTGCATACATG-3') and primers aligned within the inserted plasmid (Kan#1 5'-ATGATTGAACAAGATGGATTG-3'/Kan#2 5'-TTAATAATTCAAGAAGACTC-3' and M13F/M13R (Life Technologies) were performed as previously described (Dorella et al. 2006, Pacheco et al. 2012).

**TeO$_2$: susceptibility assays** - Potassium tellurite (K$_2$TeO$_3$) sensitivity was evaluated through determination of the minimal inhibitory concentration (MIC) by using the disk diffusion method according to the protocols of the Clinical Laboratory Standards Institute (CLSI) for antimicrobials (CLSI 2013). The MIC of K$_2$TeO$_3$ was determined using bacterial cells grown for 48 h at 37°C in trypticase soy agar (BD Difco) suspended in Müller Hinton broth (BD Difco) to a final concentration of 10$^8$ colony forming units (CFU) mL$^{-1}$ and diluted 1,000-fold into medium supplemented with various concentrations of K$_2$TeO$_3$ (Sigma-Aldrich Co) ranging from 0.0-2.5 mg/mL$^{-1}$. After incubation for 48 h at 37°C, the growth was visually assessed. The disk diffusion assay was performed with 10$^6$ CFU/mL$^{-1}$ bacteria spread onto cation-adjusted Müller-Hinton agar plates (BD Difco). The disks were then impregnated with 1 M of K$_2$TeO$_3$. After 48 h at 37°C, the diameters of the zones were determined.

**Antimicrobial susceptibility testing** - The sensitivity to antimicrobial agents (Oxoid Ltd, UK), penicillin (10 μg), erythromycin (15 μg), ampicillin (10 μg), gentamicin (10 μg), cefotaxime (30 μg), imipenem (10 μg), ciprofloxacin (5 μg), clindamycin (2 μg), rifampicin (30 μg), tetracycline (30 μg), linezolid (30 μg) and vancomycin (5 μg), was determined using the disk diffusion method according to CLSI (2013) guidelines, as previously described (Pereira et al. 2008). The inoculum equivalent to 10$^5$ colony forming units mL$^{-1}$ was determined using the disk diffusion method according to CLSI (2013). The MIC of K$_2$TeO$_3$ (Sigma-Aldrich Co) ranging from 0.0-2.5 mg/mL$^{-1}$. After incubation for 48 h at 37°C, the incubation period of 24-48 h.

**Bacterial adherence to n-hexadecane (BATH) assays** - The evaluation of bacterial adherence to n-hexadecane was performed as previously described (Mattos-Guarañal et al. 1999). Strains with BATH values > 50% were considered highly hydrophobic, 30% ≤ BATH values < 50% were considered moderately hydrophobic and BATH values < 30% were considered hydrophilic.

**Biofilm formation on glass surfaces and haemagglutination assays** - The haemagglutination activity of human B erythrocytes (0.5%) and bacterial adhesion to glass surfaces were both assayed using previously described methods (Mattos-Guarañal & Formiga 1991). The microorganisms were classified into the following categories: nonadherent (0: OD ≤ ODc), weakly adherent (+: OD < OD ≤ 2x ODc), moderately adherent (++: 2x OD < OD ≤ 4x ODc) or strongly adherent (+++: 4x OD ≤ ODc)
medium is in contact with the air, ++ (intermediate), confluent coat of cells on the sides of the tube, + (weak), LA onto the glass surface where the culture medium is in contact with air, and - (negative), no visible adherence.

**Human epithelial type 2 (HEp-2) cell interaction assays** - The cellular interaction assays were performed using epithelial cells derived from a human epidermoid larynx carcinoma (HEp-2) according to previously described protocols (Hirata Jr et al. 2002, 2004, 2008). Briefly, microorganisms grown in TSB were used to infect monolayers of HEp-2 cells grown to approximately 95% confluency. After 3 h of interaction, the infected monolayers were washed with 0.01 M PBS (pH 7.2), lysed with 0.1% Triton X-100 (Sigma-Aldrich Co) in PBS, diluted and plated. Viable bacterial counts in the supernatant and cellular monolayer lysates were subsequently determined. To determine the viable intracellular bacteria, the monolayers were treated with 150 µg mL⁻¹ of penicillin (Sigma-Aldrich Co) for 1 h. The adherence pattern assays were performed using semi-confluent HEp-2 monolayers grown on circular coverslips (13 mm diameter). At 3 h post-infection, the Giemsa-stained coverslips were examined using bright field microscopy and the observed strains were classified into the following patterns: LA, characterised by small clusters of bacteria resembling micro-colonies, diffuse adherence (DA), characterised by bacteria randomly distributed over the surfaces of the HEp-2 cells, or aggregative adherence characterised by clumps of bacteria with a “stacked-brick” appearance.

**Statistical analysis** - Each experiment was conducted in triplicate and statistical analyses were performed with the appropriate tests using a GraphPad Prism 5.0 (GraphPad, USA). p < 0.05 was considered significant.

**RESULTS**

**CDCE8392_0813 protein represented a Te₈ protein TehA homolog** - The structure and function of the putative Te₈ protein in *C. diphtheriae* CDC-E8392 (CDCE8392_0813 protein) were predicted using a protein homology/analogy recognition engine (Phyre). The best model selected by the Phyre2 server is displayed in Fig. 1A. The 302-residue protein was modelled with 100% confidence and 84% coverage (302 residues of the sequence). According to analyses of this protein, the sequence represented a Te₈ protein TehA homolog. The highest conserved region, shown in red in Fig. 1B, is likely part of the active protein site.

**CDCE8392_0813 interruption rendered the mutants more susceptible to K₂TeO₃ and H₂O₂ toxicity, but did not modify antimicrobial susceptibility profiles** - The Te₈ resistance level of the mutant LDCIC-L1 (CDC-E8392::pCR2.1-TOPO’CDC-E8392_813”), constructed via homologous recombination, was determined and compared with that of the WT strain (CDC-E8392). An increase (2x) in the susceptibility to TeO₂⁻ was observed for the mutant (MIC = 0.3125 mg/mL⁻¹). Furthermore, in disk diffusion assays, LDCIC-L1 presented a 27.00 mm (± 0.41) growth inhibition zone, which was significantly different (p = 0.0006) from the 23 mm (± 1.15) diameter inhibition zone of the WT strain. A significant difference was also observed for the sensitivity to H₂O₂ (p = 0.0019). The WT strain presented a 22.00 mm (± 0.82) growth inhibition zone, while the mutant presented a 24.70 mm (± 0.64) inhibition zone (Table II).

**Antimicrobial susceptibility assays demonstrated identical profiles for C. diphtheriae WT CDC-E8392 and LDCIC-L1 mutant strains.** Both strains were determined to be susceptible to penicillin, erythromycin, ampicillin, gentamicin, cefotaxime, imipenem, ciprofloxacin, clindamycin, rifampicin, tetracycline, linezolid and vancomycin.

**CDCE8392_0813 interruption affected the ability of C. diphtheriae to kill C. elegans and survive within HEp cells** - The results of the experiments using the nematode *C. elegans* revealed that the mutant strain LDCIC-L1 exhibited an attenuated killing ability when compared with the WT *C. diphtheriae* strain CDC-E8392 (Fig. 2).
TABLE II
Effects of Corynebacterium diphtheriae CDCE8392_0813 gene interruption on human cells interactions and on susceptibility to hydrogen peroxide

| Strain      | HA titre | Adherence pattern | Bacteria in supernatant (CFU mL⁻¹) | Adherent bacteria (CFU mL⁻¹) | Internalised bacteria (CFU mL⁻¹) | H₂O₂ inhibition halo (mm) |
|-------------|----------|-------------------|------------------------------------|----------------------------|---------------------------------|--------------------------|
| LDCIC-L1    | 32       | DA                | 11.8 x 10⁴ ± 2.23                  | 5.8 x 10⁴ ± 1.94           | 7.0 x 10⁴ ± 0.05⁻              | 24.75 ± 0.64⁻            |
| CDC-E8392   | 32       | DA                | 11.3 x 10⁴ ± 1.11                  | 5.3 x 10⁴ ± 1.30           | 10.0 x 10⁴ ± 0.57             | 22.00 ± 0.82            |

a: p < 0.05 according to unpaired t test; CFU: colony forming units; DA: diffuse adherence; HA: agglutination of human erythrocytes (type B).

DISCUSSION

A number of genetic Te⁺ determinants have been identified in different bacterial species (Taylor et al. 1994, Liu & Taylors 1999, Topetchieva et al. 2003). One of the chromosomal Te⁺ determinants is the operon tehAB, originally described in E. coli. Homologues and orthologues of the tehA gene have been identified in other bacterial species, such as Vibrio cholerae, Klebsiella pneumoniae, Salmonella enterica and Corynebacterium glutamicum (Chasteen et al. 2009, Pei et al. 2013). Recent studies have revealed that this determinant did not confer the Te⁺ mechanism of V. cholerae C6706, but was found involved in antibiotic resistance and intestinal colonisation (Pei et al. 2013).

For C. diphtheriae, a protein with similar sequence to TehA in other species was identified. This protein, referred to as CDCE8392_0813 in CDC-E8392 strain, was predicted as a Te⁺ protein TehA homologue using Phyre software. In the present study, the contribution of Te⁺ to C. diphtheriae pathogenesis was verified using the CDCE8392_0813 mutant (LDCIC-L1) constructed through chromosomal disruption. According to Phyre, the interrupted region of the CDCE8392_0813 gene likely corresponded to the protein active site because it contained many conserved residues. Gene complementation was not performed in the present study and might be considered to be a limitation of this work.

The influence of the CDCE8392_0813 protein in the Te⁺ mechanism of strain CDC-E8392 was also documented in the present study. Two different protocols used to investigate the viability of LDCIC-L1 in the presence of TeO₂⁻ revealed that this gene interruption rendered the mutant more susceptible to this compound. It has been previously suggested that Te⁺ depends on the expression of different enzymes involved in several bacterial functions, including nitrate reduction, oxidative stress response and phosphate and cysteine metabolism (Taylor 1999, Chasteen et al. 2009, Franks et al. 2014). However, the data obtained in the present study suggest that CDCE8392_0813 participates in C. diphtheriae Te⁺ and that this mechanism relies on other bacterial factors. The data also indicate that additional studies are needed to identify and characterise these other bacterial factors.

Fig. 2: Caenorhabditis elegans slow killing assays on nematode growth medium agar. The Kaplan-Meier statistical test was used to determine the probability of nematode survival. Survival curves were compared using the log rank test and considered statistically different (p < 0.05).
A recent report indicated that TehA protein participates in *V. cholerae* antimicrobial resistance (Pei et al. 2013). In contrast, the findings presented here indicated that the TehA homologue is not involved in the susceptibility of *C. diphtheriae* to the antimicrobial agents tested.

It has previously been suggested that TeO$_{2}^{2-}$ toxicity results, at least in part, from the generation of ROS including H$_2$O$_2$. However, TeO$_{2}^{2-}$ resistance is likely mediated via resistance to oxidative damage rather than the detoxification of the metal oxide itself (Topchchieva et al. 2003, Chasteen et al. 2009, Whitby et al. 2010). Due to this, the CDCE83912_0813 mutant was further examined to determine its resistance to H$_2$O$_2$. This mutant exhibited increased sensitivity to tellurite. Similar results were observed with a *Haemophilus influenzae* mutant for tehB (Whitby et al. 2010).

Because the participation of TehA in bacterial pathogenesis has been described for other species, we evaluated the involvement of the putative Te$_B$ determinant CDCE8392_0813 in the virulence of diphtheria bacilli. Using *C. elegans* as an infection model, we considered a simple but versatile animal model for analysing the virulence of bacteria, including *C. diphtheriae* (Broadway et al. 2013). Herein, we demonstrated an attenuated ability of the LDCIC-L1 mutant to kill nematodes. Similarly, mutations in the Te$_B$ genes of *Bacillus anthracis* (yce-GH) also reduced survival in *C. elegans* and increased susceptibility to TeO$_{2}^{2-}$ compounds (Franks et al. 2014).

Despite the medical relevance of *C. diphtheriae*, only a few virulence factors have been characterised in detail. In addition to diphtheria toxin, adherence factors such as glycoconjugates, haemagglutinin and pili have been well studied. These factors might be involved in biofilm formation on abiotic surfaces and/or adherence to HEP cells and extracellular components (Mattos-Guaraldi et al. 2000, Colombo et al. 2001, Ott et al. 2010, Sabbadini et al. 2010, Antunes et al. 2015). Although these studies have demonstrated that a Te$_B$ determinant might assist *E. coli* O157:H7 in establishing an infection through participation in the adherence to eukaryotic cells (Yin et al. 2009), the data obtained in the present study showed that CDCE8392_0813 protein did not influence the adherence of *C. diphtheriae* to epithelial cells. Furthermore, these results revealed that CDCE8392_0813 did not act as an adhesin or modify the expression of bacterial factors involved in *C. diphtheriae* adhesion to abiotic surfaces.

The internalisation and intracellular survival of *C. diphtheriae* strains have been demonstrated for different human cells, including epithelial HEP-2 cells (Hirata Jr et al. 2002), Detroit 562 cell line (Bertuccini et al. 2004), human umbilical vein endothelial cells (Peixoto et al. 2014) and macrophase U937 cells (Santos et al. 2010). However, the mechanisms responsible for this property are not fully understood. These data showed that the intracellular survival of *C. diphtheriae* was reduced after the interruption of the CDCE8392_0813 gene. Because LDCIC-L1 showed increased H$_2$O$_2$ sensitivity, it is likely that this determinant also contributes to *C. diphtheriae* resistance to eukaryotic intracellular defences, such as the production of ROS, in addition to bacterial resistance to TeO$_{2}^{2-}$ toxicity.

The CDCE8392_0813 gene interruption increased susceptibility to TeO$_{2}^{2-}$ toxicity, indicating that the putative Te$_B$ protein (CDCE8392_0813) (TehA) might act as the factor responsible for the expression of Te$_B$ in *C. diphtheriae* strains. Furthermore, Te$_B$ determinant might contributes to the pathogenesis of this species, as a direct correlation was verified between the expression of the CDCE8392_0813 gene and the abilities of *C. diphtheriae* to survive within the intracytoplasmic compartments of HEP cells and to kill the nematode *C. elegans*. Finally, the ability to kill the nematode *C. elegans* was verified.

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