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 (Avazéri et al. 1997, Aradská et al. 2013). Tellurite (TeO$_3^{2-}$), one of the most oxidised and soluble forms of Te, is toxic to both prokaryotes and eukaryotes. Among prokaryotes, 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, TeO$_3^{2-}$ 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 TeO$_3^{2-}$-resistance (Te$^6$), numerous plasmid and/or chromosomally encoded Te$^6$ determinants have been identified in different bacterial species, including human pathogens. The presence of Te$^6$ 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. 1995), 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).
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 (CDCE8392_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 TeO₃⁻ 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 CDCE8392_0813 gene was amplified via polymerase chain reaction (PCR) with the following primer pair: 5'-TCGGGGATGCGGGTG-3' and 5'-GGTGTGCGCAATCTGATG-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'-deoxythymidinie of linearised pCR2.1-TOPO vector provided in the kit. The resulting plasmid “pCR2.1-TOPO-CDCE8392_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'-TCGTATCAATGGCGTCCACAG-3' and 5'-AATCCTGCGGTCCCATACATG-3') and primers aligned within the inserted plasmid [Ken#1 5'-ATGATGAAAAGGATGATG-3'/Ken#2 5'-TTAATAATCCAGAGACTC-3' and M13f/M13r (Life Technologies)] were performed as previously described (Dorella et al. 2006, Pacheco et al. 2012).

**TeO₄²⁻ susceptibility assays** - Potassium tellurite (K₂TeO₄) 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 K₂TeO₄ was determined using bacterial cells grown for 48 h at 37°C in trypticase soy agar (BD DifcoTM) suspended in Müller Hinton broth (BD DifcoTM) to a final concentration of 10⁸ colony forming units (CFU) mL⁻¹ and diluted 1,000-fold into medium supplemented with various concentrations of K₂TeO₄ (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 10⁶ CFU mL⁻¹ bacteria spread onto cation-adjusted Müller-Hinton agar plates (BD DifcoTM). The disks were then impregnated with 1 M of K₂TeO₄. 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 a 0.5 McFarland standard was placed on the surface of a cation-adjusted Müller-Hinton agar containing 5% sheep blood. The plates were subsequently incubated at 37°C for 24 h and reconfirmed at 48 h. Breakpoints for the susceptible strains were used in accordance with the CLSI for bacteria excluded from table 2A-J. Because there is no defined standard for interpreting these results, the results were interpreted in accordance with (CLSI 2010). The breakpoints for S. aureus, established by CLSI, were considered in the cases of penicillin.

**Agar diffusion growth inhibition assays for susceptibility to H₂O₂** - The sensitivity to H₂O₂ was assessed based on the methods of Kim and Holmes (2012) with some adaptations. Approximately 10⁶ CFU mL⁻¹ were spread onto the surface of cation-adjusted Müller-Hinton agar plates. A 10-µL aliquot of 20 mM H₂O₂ was spotted onto the centre of the plate and the diameter of the growth inhibition zone was measured after an incubation period of 24-48 h.

**Nematode infection model** - The assays were performed as previously described with some adaptations (Browning et al. 2013). Briefly, Caenorhabditis elegans N2 was maintained on plates containing nematode growth medium (NGM) agar for approximately six-seven days at 20°C and used in infection assays with wild-type (WT) and mutant (LDCIC-L1) C. diphtheriae strains. Twenty L4 stage larval worms were infected with 20 µL of each bacterial strain (obtained from an overnight culture) on NGM plates at 20°C for 24 h. The worms were assessed daily following infection and the dead nematodes were counted and removed every 24 h for seven days. For each strain, approximately 60 nematodes were used and the assays were performed three times. E. coli OP50 was used as a control in these experiments.

**Biofilm formation on polystyrene surfaces** - Biofilm formation was determined in 96 well flat-bottomed polystyrene microtitre plates based on previously described methods (Gomes et al. 2009). Briefly, 200 µL of the bacterial suspension with an optical density (OD) of 0.2 (λ = 570 nm) in TSB medium was applied to each well of the microplate. For the negative control, only TSB was applied to the well. After an incubation period of 48 h at 37°C, the content of each well was aspirated and washed with 0.01 M phosphate-buffered saline (PBS) (pH 7.2). The remaining attached bacteria were fixed with 99% methanol and stained with 2% v/v crystal violet. The bound dye was subsequently solubilised using 33% glacial acetic acid and the OD of the solution was measured at 570 nm using an enzyme immunosorbent assay reader (BioRad Laboratories Inc; model 550). The cut-off OD (ODc) was defined as the mean OD of the negative control. The strains were classified into the following categories: nonadherent (0: OD ≤ ODc), weakly adherent (+: ODc < OD ≤ 2x ODc), moderately adherent (++: 2x ODc < OD ≤ 4x ODc) or strongly adherent (+++: 4x ODc ≤ OD).

**Bacterial adherence to n-hexadecane (BATH) assays** - The evaluation of bacterial adhesion to n-hexadecane was performed as previously described (Mattos-Guaraldi 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-Guaraldi & Formiga 1991). The microorganisms were classified into the following categories for their ability to adhere onto glass: +++ (strong), confluent coat of cells on the sides of the tube and localised adherence (LA) onto the glass surface where the culture
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% confluence. 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 Te² protein TehA homolog - The structure and function of the putative Te² 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 Te² 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 Te² 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 TeO₃²⁻ 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). The
data shown in Table II also indicated a reduced ability of the mutant to survive in the intracytoplasmic compartment of HEp-2 cells (p = 0.0391 according to t test).

Strains CDC-E8392 and LDCIC-L1 reached internalisation rates of 1.89 ± 0.15% and 1.21 ± 0.35%, respectively.

CDCE8392_0813 interruption did not affect the hydrophobicity and adherence properties of C. diphtheriae. The ability of C. diphtheriae LDCIC-L1 to adhere to and to form a biofilm on abiotic surfaces was evaluated using glass and polystyrene adherence assays. Simultaneously, the surface hydrophobicity of this strain was analysed through BATH assays and haemagglutinating activity. The mutant showed nonadherence to the glass and moderate adherence to polystyrene surfaces (Table I). The mutant also preferred a hydrophobic surface (% BATH = 51) (Table I) and the displayed haemagglutinating activity (titre = 32) (Table II).

The adherence of the mutant strain to HEp cells was verified using the HEp-2 lineage; the results are displayed in Table II. Similar to the WT strain, the mutant strain showed a DA pattern with a moderate degree of adherence (31.9%) after 3 h of interaction. The adherence values were not considered to be significantly different from the parental strain according to unpaired t test (p > 0.01).

### DISCUSSION

A number of genetic Te<sup>8</sup> determinants have been identified in different bacterial species (Taylor et al. 1994, Liu & Taylor 1999, Toptchieva et al. 2003). One of the chromosomal Te<sup>8</sup> 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<sup>8</sup> 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<sup>8</sup> protein TehA homologue using Phyre software. In the present study, the contribution of Te<sup>8</sup> 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<sup>8</sup> 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 TeO<sub>3</sub><sup>2-</sup> revealed that this gene interruption rendered the mutant more susceptible to this compound. It has been previously suggested that Te<sup>8</sup> 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<sup>8</sup> 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.
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$_2^-$ toxicity results, at least in part, from the generation of ROS including H$_2$O$_2$. However, TeO$_2^-$ resistance is likely mediated via resistance to oxidative damage rather than the detoxification of the metal oxide itself (Topchieva 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$^6$ 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$^6$ genes of Bacillus anthracis (ycfGH) also reduced survival in C. elegans and increased susceptibility to TeO$_2^-$ 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$^6$ 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 macrophage 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$_2^-$ toxicity.

The CDCE8392_0813 gene interruption increased susceptibility to TeO$_2^-$ toxicity, indicating that the putative Te$^6$ protein (CDCE8392_0813) (TehA) might act as the factor responsible for the expression of Te$^6$ in C. diphtheriae strains. Furthermore, Te$^6$ determinant might contribute 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.

REFERENCES


