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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Corynebacterium diphtheriae, *the aetiologic agent of diphtheria, also represents a global medical challenge because of the existence of invasive strains as causative agents of systemic infections. Although tellurite (TeO_3^{2-}) is toxic to most microorganisms, TeO_3^{2-}-resistant bacteria, including* C. diphtheriae, *exist in nature. The presence of TeO_3^{2-}-resistance (Te^R) determinants in pathogenic bacteria might provide selective advantages in the natural environment. In the present study, we investigated the role of the putative Te^R determinant (CDCE8392_813 gene) in the virulence attributes of diphtheria bacilli. The disruption of CDCE8392_0813 gene expression in the LDCIC-L1 mutant increased susceptibility to TeO_3^{2-} and reactive oxygen species (hydrogen peroxide), but not to other antimicrobial agents. The LDCIC-L1 mutant also showed a decrease in both the lethality of* Caenorhabditis elegans *and the survival inside of human epithelial cells compared to wild-type strain. Conversely, the haemagglutinating activity and adherence to and formation of biofilms on different abiotic surfaces were not regulated through the CDCE8392_0813 gene. In conclusion, the CDCE8392_813 gene contributes to the Te^R and pathogenic potential of* C. diphtheriae.

Key words: *Corynebacterium diphtheriae* - tellurite-resistance - CDCE8392_0813 protein - HEp-2 cells - *Caenorhabditis elegans*

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^R), numerous plasmid and/or chromosomally encoded Te^R determinants have been identified in different bacterial species, including human pathogens. The presence of Te^R 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 Te[R] 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 Te[R] 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 (H_2O_2), 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 37ºC 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 37ºC. When appropriate, kanamycin (Sigma-Aldrich Co, USA) was added (50 μg mL^{-1}).

In silico search and three-dimensional model prediction of the putative Te[R] 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 TeO_3^{2-} resistance/dicarboxylate transporter family was identified in all sequenced strains.

The Phyre2 server was used to predict the three-dimensional structure of the putative Te[R] 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 Te[R] gene - For the chromosomal disruption of the *C. diphtheriae* putative Te[R] 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'-TCGTTTTATGCGGGTGC-3' and 5'-GGTGT-GCGCAATCTGATG-3'. Amplification was performed with an initial denaturation step at 94ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min and extension at 72ºC for 1 min and 30 s and a final elongation step at 72ºC for 10 min.

The DNA fragment was ligated to the overhanging 3'-deoxythymidine 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 Miniprep Kit (Thermo Fisher Scientific Inc Invitrogen™). Five

TABLE I
Biological properties of *Corynebacterium diphtheriae* strains used in this study

Strain	Origin	Biovar/sucrose fermentation	*tox* gene[a]	*tehA*	K_2TeO_3 MIC (mg.mL^{-1})	K_2TeO_3 inhibition halo (mm)	BATH (%)	Glass/polystyrene adherence	HEp-2 cells adherence pattern	References
LDCIC-L1	CDC-E8392::pCR2.1-TOPO'CDC-E8392_813''	mitis/suc -	+	Interrupted	0.31	27 ± 0.41[b]	51 ± 0.82	- /++	DA	This paper
CDC-E8392	Diphtheria/pharynx (USA)	mitis/suc -	+	+	0.62	23 ± 1.15	51 ± 1.20	- /++	DA	Mattos-Guaraldi et al. (1999), Hirata Jr et al. (2002, 2004), Moreira et al. (2003), Gomes et al. (2009)

a: toxicity evaluated by polymerase chain reaction for *tox* gene (Pimenta et al. 2008); *b*: p < 0.05 according to unpaired *t* test; BATH: bacterial adherence to *n*-hexadecane; CDC: Centres for Disease Control and Prevention; DA: diffuse adherence [characterised by bacteria randomly distributed over the surfaces of the human epithelial type 2 (HEp-2) cells]; K_2TeO_3: potassium tellurite; MIC: minimal inhibitory concentration; -: negative; +: positive; ++: moderately adherent to glass or polystyrene.

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 37ºC. The culture was plated onto a TSB agar plate containing kanamycin and maintained at 37ºC 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 CDCE8392_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 CDCE8392_0813 gene (5'-TCGTATCAAT-GCGTGCCACAG-3' and 5'-AATCGTGCGCTGCCAT-ACATG-3') and primers aligned within the inserted plasmid [Kan#1 5'-ATGATTGAACAAGATGGATTG-3'/ Kan#2 5'-TTAATAATTCAGAAGAACTC-3' and M13F/ M13R (Life Technologies)] were performed as previously described (Dorella et al. 2006, Pacheco et al. 2012).

TeO$_3^{2-}$ 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 37ºC 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 37ºC, the growth was visually assessed. The disk diffusion assay was performed with 10^8 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 37ºC, 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 37ºC 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 H$_2$O$_2$ - The sensitivity to H$_2$O$_2$ was assessed based on the methods of Kim and Holmes (2012) with some adaptations. Approximately 10^8 CFU mL^{-1} were spread onto the surface of cation-adjusted Müller-Hinton agar plates. A 10-µL aliquot of 20 mM H$_2$O$_2$ 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 20ºC 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 20ºC 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 37ºC, 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 $\leq$ OD$_c$), weakly adherent (+: OD$_c$ < OD $\leq$ 2x OD$_c$), moderately adherent (++: 2x OD$_c$ < OD $\leq$ 4x OD$_c$) or strongly adherent (+++: 4x OD$_c$ $\leq$ 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% $\leq$ 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
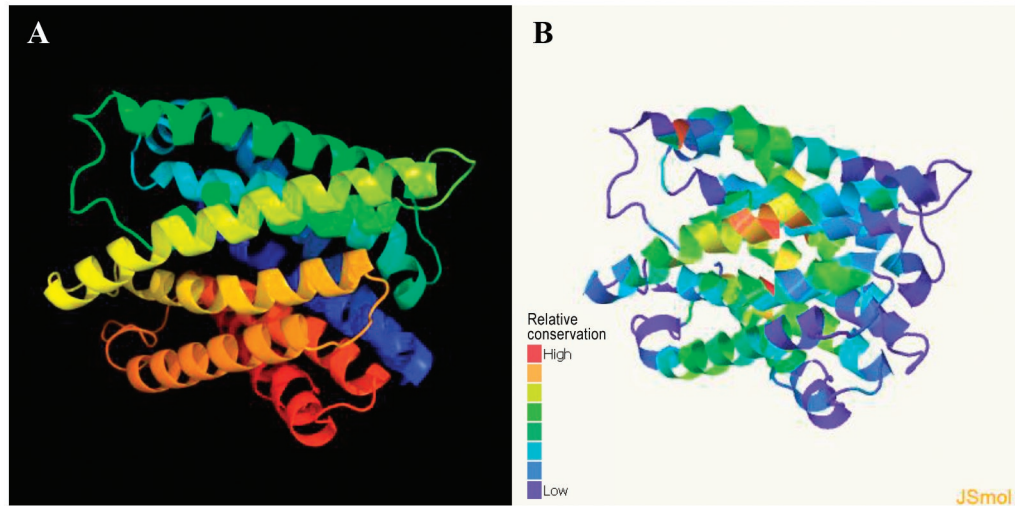
Fig. 1: three-dimensional models provided by Phyre2 server. A: best model for the CDCE8392_0813 protein (100% confidence and 84% coverage); B: conserved regions of CDCE8392_0813 protein.

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 Te^R protein TehA homolog - The structure and function of the putative Te^R 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 Te^R 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 K$_2$TeO$_3$ and H$_2$O$_2$ toxicity, but did not modify antimicrobial susceptibility profiles - The Te^R 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 TeO$_3$$^{2-}$ 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 H$_2$O$_2$ (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

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 | Human epithelial type 2 epithelial cell | | | |
			Bacteria in supernatant (CFU mL^{1-})	Adherent bacteria (CFU mL^{1-})	Internalised bacteria (CFU mL^{1-})	H$_2$O$_2$ inhibition halo (mm)
LDCIC-L1	32	DA	$11.8 \times 10^4 \pm 2.23$	$5.8 \times 10^4 \pm 1.94$	$7.0 \times 10^2 \pm 0.05^a$	24.75 ± 0.64^a
CDC-E8392	32	DA	$11.3 \times 10^4 \pm 1.11$	$5.3 \times 10^4 \pm 1.30$	$10.0 \times 10^2 \pm 0.57$	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).
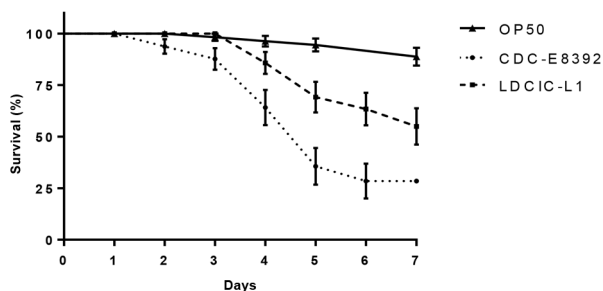

Fig. 2: *Caenorhabditis elegans* slow killing assays on nematode growth medium agar. The Kaplan-Meier statistical test was used to determine the probability of nematode survival. Survival curves were compared using the log rank test and considered statistically different ($p < 0.05$).

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 $1.89 \pm 0.15\%$ and $1.21 \pm 0.35\%$, 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^R determinants have been identified in different bacterial species (Taylor et al. 1994, Liu & Taylor 1999, Toptchieva et al. 2003). One of the chromosomal Te^R determinants is the operon *teh*AB, originally described in *E. coli*. Homologues and orthologues of the *teh*A 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^R 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^R protein TehA homologue using Phyre software. In the present study, the contribution of Te^R 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^R 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$_3^{2-}$ revealed that this gene interruption rendered the mutant more susceptible to this compound. It has been previously suggested that Te^R 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^R 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_3^{2-} toxicity results, at least in part, from the generation of ROS including H_2O_2. However, TeO_3^{2-} resistance is likely mediated *via* resistance to oxidative damage rather than the detoxification of the metal oxide itself (Toptchieva 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_2O_2. This mutant exhibited increased sensitivity to tellurite. Similar results were observed with a *Haemophilus influenzae* mutant for *teh*B (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^R 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^R genes of *Bacillus anthracis* (*yce-GH*) also reduced survival in *C. elegans* and increased susceptibility to TeO_3^{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^R 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_2O_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_3^{2-} toxicity.

The CDCE8392_0813 gene interruption increased susceptibility to TeO_3^{2-} toxicity, indicating that the putative Te^R protein (CDCE8392_0813) (TehA) might act as the factor responsible for the expression of Te^R in *C. diphtheriae* strains. Furthermore, Te^R 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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