Characterization of DIP0733, a multi-functional virulence factor of Corynebacterium diphtheriae

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Corynebacterium diphtheriae is typically recognized as an extracellular pathogen. However, a number of studies revealed its ability to invade epithelial cells, indicating a more complex pathogen–host interaction. The molecular mechanisms controlling and facilitating internalization of Cor. diphtheriae are poorly understood. In this study, we investigated the role of DIP0733 as virulence factor to elucidate how it contributes to the process of pathogen–host cell interaction. Based on in vitro experiments, it was suggested recently that the DIP0733 protein might be involved in adhesion, invasion of epithelial cells and induction of apoptosis. A corresponding Cor. diphtheriae mutant strain generated in this study was attenuated in its ability to colonize and kill the host in a Caenorhabditis elegans infection model system. Furthermore, the mutant showed an altered adhesion pattern and a drastically reduced ability to adhere and invade epithelial cells. Subsequent experiments showed an influence of DIP0733 on binding of Cor. diphtheriae to extracellular matrix proteins such as collagen and fibronectin. Furthermore, based on its fibrinogen-binding activity, DIP0733 may play a role in avoiding recognition of Cor. diphtheriae by the immune system. In summary, our findings support the idea that DIP0733 is a multi-functional virulence factor of Cor. diphtheriae.

INTRODUCTION

The genus Corynebacterium belongs to the class of Actinobacteria (i.e. Gram-positive bacteria with high G + C DNA content) and comprises a collection of irregular- or club-shaped (micro)aerobic bacteria (Ventura et al., 2007; Zhi et al., 2009). Today, 88 Corynebacterium species are described with about 53 having a more or less pronounced medical importance (Bernard, 2012). The most prominent and important member of the pathogenic species is Corynebacterium diphtheriae, which is also the type species of the genus.

Cor. diphtheriae is the classical aetiological agent of diphtheria (Hadfield et al., 2000; Burkovski, 2013a, b), a localized toxaeamic infection of respiratory tract that can be fatal. Moreover, it can cause skin ulcers (cutaneous diphtheria) as well as systemic infections such as endocarditis, meningitis, pneumonia and others (Murphy, 1996; Burkovski, 2013b). This indicates that Cor. diphtheriae is able to colonize not only epithelia but also deeper parts of the body and that the bacteria interact with various types of host cells. The ability of Cor. diphtheriae to enter cultured respiratory epithelial cells was first shown by Hirata et al. (2002) and validated by a number of further studies (Mattos-Guaraldi, 2002; Bertuccini et al., 2004; Ott et al., 2010a, b, 2013).

Due to its medical importance, Cor. diphtheriae might be the best-investigated pathogenic member of the genus; however, even for this species only a few virulence factors have been characterized in detail. Beside the diphtheria toxin these include mainly pili and a few other adhesion factors (see Collier, 2001; Rogers et al., 2011; Readon-Robinson & Ton-That, 2013; Ott & Burkovski, 2013 for reviews), while little is known about the factors mediating the entry processes in the host cell and the receptors...
recognized. An interesting protein in respect to its role in *Cor. diphtheriae* pathogenicity is DIP0733, which was initially described as non-fimbrial surface protein 67-72p according to its appearance as two bands with distinct apparent masses in SDS-PAGE (Colombo et al., 2001). Both the 67 and 72 kDa polypeptide bind to erythrocyte receptors, leading to haemagglutination. Subsequently, Hirata et al. (2004) demonstrated that 67-72p binds not only human erythrocytes but also the human epidermoid laryngeal carcinoma cell line HEp-2 and that binding was effectively blocked by anti-67-72p IgG antibodies. A recent MS study indicated that the 67-72p protein is encoded by a single gene, *dip0733* (Sabbadini et al., 2012). Furthermore, an effect of the protein on internalization and induction of apoptosis was suggested based on experiments using 67-72p protein-coated latex beads (Sabbadini et al., 2012). Despite the different properties attributed to the protein, i.e. haemagglutination, binding to host cell receptors and induction of apoptosis, the protein sequence is lacking obvious functional domains. As a further step of a molecular characterization of this functionally astonishingly diverse protein, we studied the interaction of 67-72p wild-type and mutant strains with *Caenorhabditis elegans*, an invertebrate model system, as well as with epithelial cells and macrophages. In addition, we investigated if DIP0733 is involved in interactions with extracellular matrix components fibronectin and type I collagen and with human plasma fibrinogen. In summary, our results indicate that DIP0733 can be considered as a microbial surface components recognizing adhesive matrix molecule (MSCRAMM) with virulence properties.

**METHODS**

**Reconstruction of phylogenetic trees.** Sequences with amino acid identities of 45% or higher for multiple alignment were obtained by a BLAST of DIP0733 sequence versus the UniProtKB database using default values. The multiple alignment was performed by CLUSTAL W 2.0 (Larkin et al., 2007). Phylogenetic trees were calculated by the neighbour-joining method using the Jones–Taylor–Thirnton distance correction was used as indicated.

**Bacterial strains and culture conditions.** Strains used in this study are listed in Table 1. *Escherichia coli* OP50 and *Top10 Electrocomp* were grown in Luria–Bertani (LB) medium at 37 °C (Sambrook et al., 1989). *Cor. diphtheriae* strains were grown in heart infusion (HI) broth or trypticase soy broth (TSB) at 37 °C and stored in TSB medium with 20% glycerol at −80 °C. When appropriate, kanamycin was added (50 μg kanamycin ml⁻¹ final concentration).

**Molecular biology methods.** Standard techniques were used for plasmid isolation, transformation and cloning (Sambrook et al., 1989). For chromosomal disruption of the *Cor. diphtheriae* DIP0733 gene, a 1068 bp internal DNA fragment was amplified by PCR. Chromosomal DNA of strain CDC-E8392 was used as template and the following primers: 5'-CACCCAGGCCTTCCTCGTTTTT-3' and 5'-GGCGGCGGCCCGGTTACTGC-3'. The DNA fragment was ligated to the overhanging 3' deoxythymidine (T) of pCR2.1-TOPO linearized vector exploiting the Tαg polymerase's non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The resulting plasmid pCR2.1-TOPO-DIP0733 was used to transform *E. coli* TOP10 Electrocomp cells. Subsequently, 1 μg unmethylated plasmid DNA isolated from this *E. coli* strain was used to transform *Cor. diphtheriae* using a GenePulsar II apparatus (Bio-Rad). Electroporated cells were added to 1 ml TSB and incubated for 4 h at 37 °C. This culture was plated on TSB agar plates containing kanamycin. Since pCR2.1-TOPO cannot autonomously replicate in *Cor. diphtheriae*, kanamycin-resistant *Cor. diphtheriae* carried the vector integrated via homologous recombination in the chromosomal CDC-E8392_B0678 gene. The insertion of the plasmid into the chromosome of *Cor. diphtheriae* CDC-E8392 strain was confirmed by PCR employing different combinations of primers and Southern blotting (see Fig. S1, available in the online Supplementary Material). The confirmed strain was designated CAM-1.

For overexpression and complementation, the complete DIP0733 gene sequence was amplified by PCR (primers: 5'-CGCGGCTCTAGAATTC-3' and 5'-CGCGGCCGCGGGTTACTGC-3') and the product was digested with the restriction enzymes BstI and XmaI for 1.5 h at 37 °C. The vector pXM119 (Jakoby et al., 1999) was digested with the same enzymes in parallel and dephosphorylated with Rapid Phosphatase (Roche) for 30 min at 37 °C. Ligation of the insert into the pXM119 plasmid was carried out with T4 DNA ligase overnight at room temperature. After transformation of *E. coli* DH5α MCR, the positive clones were selected on LB medium containing 25 μg chloramphenicol ml⁻¹. The resulting plasmid pXM119-DIP0733 was isolated, DNA sequenced and transformed in *Cor. diphtheriae* as described above. For verification of DIP0733 transcription, see Fig. S2.

For fluorescence microscopy experiments, plasmid pXM119-DIP0733-mCherry was constructed carrying the DIP0733 gene between the tac promoter and mCherry. For this purpose, a 0.7 kb KpnI/EcoRI DNA fragment carrying the mCherry gene was isolated from pXM119mCherry and ligated downstream of the DIP0733 gene in plasmid pXM119-DIP0733.

**Adherence and internalization assays.** HeLa cells were seeded in 24-well plates (Nunc) with 5 × 10⁴ cells per well 48 h prior to infection. Bacteria were inoculated to an OD₆₀₀ of 0.1 from overnight cultures and grown in HI broth to an OD₆₀₀ of 4.0 to 0.6. Subsequently, the bacteria were harvested by centrifugation and cell density was adjusted to an OD₆₀₀ of 0.5. A master mix of the inoculum with a m.o.i. of 50 was prepared in Dulbecco’s modified Eagle’s medium (DMEM) and 500 μl per well was used to infect the cells. The plates were centrifuged for 5 min at 500 r.p.m. to synchronize infection and subsequently incubated for 90 min. The cells were washed with PBS three times, detached with 500 μl Accutase per well (5 min at 37 °C with 5 % CO₂ and 95% humidity) and lysed with 0.025 % Tween 20 under same conditions. Serial dilutions were made in pre-chilled 1 % PBS and plated on Columbia agar with sheep blood (Oxoid) to determine the number of c.f.u.

For invasion analysis, the cells were washed three times with PBS to remove planktonic and loosely attached bacteria. Subsequently, the cells were incubated for 2 h in DMEM (500 μl per well), containing 100 μg gentamicin ml⁻¹ to kill remaining extracellular bacteria. After this incubation, the cell layers were washed three times with PBS, detached by adding 500 μl trypsin solution (0.12 % trypsin, 0.01 % EDTA in PBS) per well (5 min at 37 °C with 5 % CO₂ and 95% humidity) and lysed for 5 min at 37 °C with 0.025 % Tween 20 to liberate the intracellular bacteria. Serial dilutions of the inocula and the lysates were plated out on Columbia agar with sheep blood (Oxoid) to determine the number of c.f.u.

**Infection of THP-1 cells.** Human THP-1 cells were cultured in 10 % PBS supplemented RPMI medium 1640 (containing 100 U penicillin ml⁻¹ and 100 mg streptomycin ml⁻¹) at 37 °C in 5 % CO₂ in a
humidified cell culture incubator. Before infection, THP-1 cells were cultured in antibiotic-free medium containing 10% FBS and were differentiated into macrophage-like cells using 10 ng PMA ml−1 for 24 h. The cells were infected with Cor. diphtheriae CDC-E8392 and the corresponding mutant CAM-1 at m.o.i. of 10 or left uninfected. The plates were centrifuged for 5 min at 500 r.p.m. to synchronize infection. After incubation for 30 min, the medium was aspirated and the plates were centrifuged for 5 min at 500 r.p.m. to synchronize infection. After incubation for 30 min, the medium was aspirated and the cells were treated first with medium containing 100 μg gentamicin ml−1 for 1 h. Then, the medium with a lower gentamicin concentration (10 μg gentamicin ml−1) was added and cells were incubated until they were harvested (1, 7 or 19 h). The supernatant was removed and cells were lysed with 500 μl 0.1% Triton X-100, serial dilutions of the inocula and the lysates were plated out on Columbia agar with sheep blood (Oxoid) to determine the number of cfu.

**Infection and colonization of Cae. elegans.** Cae. elegans N2 was maintained on E. coli OP50 for 6 to 7 days until the worms became starved, as indicated by clumping behaviour (de Bono & Bargmann, 1998). Subsequently, the nematodes were infected with different Cor. diphtheriae strains. Infection of L4 stage larval worms was carried out with 20 μl of each bacterial strain (from an overnight culture) on NGM plates at 21°C for 24 h. Worms were assessed each day following infection and the dead nematodes were counted and removed every 24 h. For each strain, approximately 60 nematodes were used and the assays were performed three times. The Kaplan–Meier survival analysis was used and all statistical analyses were performed with Prism 5.0 (GraphPad), with P values of less than 0.05 considered significant.

The isolation of bacteria after colonization of worms was carried out as previously described (Garsin et al., 2001). In short, to remove the surface bacteria, the worms were washed twice in 4 ml M9 medium and transferred to new NGM plates supplemented with 25 μg nalidixic acid ml−1. Subsequently, 10 nematodes were placed in 1.5 ml reaction tubes containing 10 μl PBS with 1% Triton X-100 and were mechanically disrupted by using a pestle. Then, 200 μl of 1% Triton X-100 was added and 100 μl aliquots of the liquid were plated on HI agar with and without antibiotics.

**Extracellular matrix (ECM) and plasma protein binding assays.**

Bacterial binding to type I collagen, fibronectin and biotinylated fibrogenin (Sigma) was performed in 96-well ELISA microtitre plates (Costar 96 Well EIA/RIA plate; Corning). Bacterial cultures grown for 24 h at 37°C in TSB medium were washed twice with PBS and resuspended in 0.1 M NaHCO3, pH 9.6, to an OD600 of 0.2 (equivalent to 5×108 c.f.u. ml−1). The wells were incubated with 100 μl of bacterial suspensions for 1 h at 37°C and then overnight at 8°C. A standard curve was performed by diluting the biotinylated protein solutions to concentrations varying from 5 to 0.05 mg ml−1 (1 h at 37°C). After blocking with 2% BSA (BSA type V; Sigma) in PBS containing 0.05 Tween-20 (PBST) for 1 h at 37°C, the wells were washed three times with PBST. The bacterial strains were subsequently incubated with 20 mg biotinylated ECM/plasma proteins ml−1 at 37°C. After three washes with PBST, the wells were incubated for 30 min at 37°C with 0.001 g extravidin-peroxidase ml−1 (Sigma) prepared in PBST containing 1% BSA. After washing three times, 3,3′,5,5′-tetramethylbenzidine liquid substrate was added. After 20 min the reaction was stopped with 50 μl of 1 M HCl and colour development was measured at λ=450 nm in a microtitre plate reader. The colour intensity of the wells sensitized with the micro-organisms was compared to the standard curve by GraphPad Prism, version 6.0. The results were expressed in micrograms of adhered proteins, with a mean ± SD of three independent assays, each performed in triplicate. The mean of the binding was compared to the standard curve by Tukey’s multiple comparison test (Simpson-Louredo et al., 2014).

**RESULTS**

**Distribution of DIP0733 genes.**

Using MALDI-TOF analyses 67-72p was identified with significant scores as the protein DIP0733 (Sabbadini et al., 2012). Corresponding genes are widely distributed among members of the genera Corynebacterium, Mycobacterium.
and Nocardia (CMN group of Actinobacteria), but corresponding genes were also found in the genomes of Amycolatopsis, Dietzia and Rhodococcus species. Taken together, more than 250 homologous genes were found in genome sequence data collections, both in pathogenic and in non-pathogenic Actinobacteria. Phylogenetic analyses revealed distinct clusters of closely related members of this group within the genera Corynebacterium, Gordonia, Amycolatopsis, Nocardia and Rhodococcus, while members of the genus Mycobacterium form two distinct clusters [overview Fig. 1, see Fig. S3 for the reconstructed phylogenetic tree (1.000 actinobacterial DIP0733-like proteins extracted from databases)]. In several Mycobacterium and Rhodococcus species two or three paralogues were found (Table S1), while a number of Actinobacteria lack DIP0733 genes (Table S2).

Interaction with Cae. elegans

A number of different model systems including amoeba, nematodes, insects, fishes and mammals have been introduced to study host–pathogen interactions. Besides guinea pigs already applied by Loeffler, wax moth larvae (Galleria mellonella) and the nematode Cae. elegans were established as simple surrogate model systems for the investigation of Cor. diphtheriae pathogenicity and virulence factors (Ott et al., 2012; Broadway et al., 2013).

When Cae. elegans were fed with E. coli OP50 carrying plasmid pXMJ19mCherry, no fluorescence was detectable after the nematodes were set back on agar plates containing unlabelled E. coli, since the bacteria were unable to colonize the nematodes and were completely digested (Fig. 2a). When the nematodes were fed with mCherry-labelled Cor. diphtheriae wild-type CDC-E8392, the complete gut was fluorescent even after Cae. elegans were set back on agar plates containing unlabelled OP50 (Fig. 2b), thus indicating persistent colonization. In contrast, Cae. elegans infected with DIP0733 gene disruption mutant CAM-1 revealed an almost complete clearance of mCherry-expressing bacteria after transfer to plates with unlabelled E. coli. Only slight background fluorescence was detectable in this case, indicating a significantly impaired colonization by CAM-1 pXMJ19mCherry (Fig. 2c). This defect was complemented at least partially, when strain CAM-1 was transformed with plasmid pXMJ19-DIP0733-mCherry (Fig. 2d).

As a more quantitative assay and to avoid putative misinterpretation caused by fluorescent protein, which might remain in the gut after digestion of bacteria, c.f.u. were determined from Cae. elegans infected with Cor.

![Phylogenetic tree of the DIP0733 family of proteins. Unrooted phylogenetic trees were reconstructed with the CLUSTAL W software making use of the implemented neighbour joining method with the function for evolutionary distance correction. Evolutionary distances are proportional to the branch length.](image-url)
diphtheriae strains. After 24 h of colonization, about 10-fold higher numbers of c.f.u. were obtained from lysed worms infected with wild-type CDC-E8392 compared to mutant strain CAM-1 (254 ± 189 versus 30 ± 13 c.f.u. ml⁻¹). Moreover, the mutant bacteria were, in contrast to the wild-type, less detrimental to Cae. elegans. In a nematode survival assay, about 90% of the nematodes survived contact with the mutant strain CAM-1, while 70% were killed by the wild-type CDC-E8392 within 7 days (Fig. 3a). The mutant strain and wild-type showed enhanced nematode toxicity when carrying DIP0733 expression vector pXMJ19-DIP0733, while the empty vector control pXMJ19 had no significant influence, indicating successful complementation and overexpression of DIP0733 (Fig. 3b). In summary, disruption of the DIP0733 gene reduced host colonization and killing dramatically in the Cae. elegans model system.

**Influence on adhesion to epithelial cells**

As a more quantitative approach to characterize adhesion, the adhesion rate of wild-type CDC-E8392 and DIP0733 mutant strain CAM-1 to HeLa cells was determined. While CDC-E8392 reached an adhesion rate of 25.97 ± 7.06 %, CAM-1 attachment was reduced to only 6.93 ± 2.98 %. Similar rates were obtained with HEp-2 cells (data not shown). The rates were unchanged when the corresponding strains were transformed with Cor. diphtheriae plasmid pXMJ19 (24.72 ± 3.91 % for the wild-type and 9.43 ± 3.61 % for CAM-1), while transformation with DIP0733 expression vector pXMJ19-DIP0733 led to a significantly enhanced adhesion in wild-type CDC-E8392 and mutant strain compared to the plasmid-free strains. Overexpression of
DIP0733 led to an adhesion rate of $59.63 \pm 9.81\%$ for the wild-type and $49.87 \pm 8.81\%$ for CAM-1. Obviously, complementation of the DIP0733 mutation was possible and overexpression resulted in strongly enhanced adherence to epithelial cells (Fig. 4a).

**Binding to extracellular matrix proteins and fibrinogen**

Four main fibrous proteins are constituents of the extracellular matrix surrounding eukaryotic cells, collagen, fibronectin, laminin and elastin. Pathogenic bacteria developed so-called MSCRAMMs, which interact with these proteins (Chagnon et al., 2012). Also for *Cor. diphtheriae* an interaction with collagen and fibronectin was reported (Sabbadini et al., 2010).

To test binding to extracellular matrix components, bacteria were coupled to microtitre plate wells and incubated with type I collagen and fibronectin. Both strains adhered to the plates with the same coating efficiency as determined by Sorensen & Brodbeck (1986). Compared to the wild-type CDC-E8392, collagen binding of a DIP0733 mutant CAM-1 was significantly impaired. Binding to collagen, which might be especially crucial for *Cor. diphtheriae* strains causing septic arthritis (Puliti et al., 2006), was reduced by about 40% ($0.197 \pm 0.001 \mu g/5 \times 10^8$ of c.f.u. wild-type CDC-E8392 and $0.104 \pm 0.005 \mu g/5 \times 10^8$ c.f.u. of CAM-1). Furthermore, the binding to fibronectin was at least slightly reduced ($0.232 \pm 0.002 \mu g/5 \times 10^8$ c.f.u. of the wild-type and $0.182 \pm 0.001 \mu g/5 \times 10^8$ c.f.u. of CAM-1) (Fig. 5). In summary, DIP0733 seems to function as a *Cor. diphtheriae* MSCRAMM and the impaired adhesion to epithelial cells might be at least partially the result of disturbed ECM recognition by the mutant.

Fibrinogen is a major component of the human plasma and is crucial for blood clot formation due to its conversion into insoluble fibrin, a process that is hijacked by many pathogens. Sabbadini et al. (2010) already demonstrated that *Cor. diphtheriae* is able to bind to fibrinogen and convert it to fibrin. In an approach similar to the binding assays described above, DIP0733 disruption strain CAM-1 showed ~50% reduced binding to fibrinogen compared to the wild-type ($0.417 \pm 0.029 \mu g/5 \times 10^8$ c.f.u. of CAM-1).

**Fig. 4.** Adhesion to and invasion of epithelial cells. (a) HeLa cells were infected with *Cor. diphtheriae* strains, washed with PBS, detached with trypsin solution, and lysed with Tween 20, and the number of c.f.u. was determined. Adhesion is expressed as percentage of the inoculum, showing means and SD of 22, 6 and 8 independent measurements (biological replicates) with three samples each (technical replicates) for the strains indicated. (b) Invasion of epithelial cells. HeLa cells were infected for 2 h with *Cor. diphtheriae* strains, treated with 100 µg gentamicin ml$^{-1}$, washed with PBS, detached with trypsin solution, and lysed with Tween 20, and the number of c.f.u. was determined. Invasion is expressed as percentage of the inoculum, showing means and SD of 22, 6 and 8 independent measurements (biological replicates) with three samples each (technical replicates) for wild-type and mutant strains without plasmid, transformed with the empty plasmid and overexpression plasmid, respectively. Statistically relevant differences between the strains (based on Student’s t-test) are indicated by asterisks above columns (**$P<0.005$ and *$P<0.05$). ND, Not determined.

**Fig. 5.** Binding to extracellular matrix proteins and fibrinogen. Binding to type I collagen, fibronectin and human fibrinogen by *Cor. diphtheriae*. Black and white bars represent CDC-E8392 and CAM-1, respectively. For statistic evaluation, see legend to figure 4.
of the wild-type and $0.192 \pm 0.003 \mu g/5 \times 10^8$ c.f.u. of CAM-1 (Fig. 5), indicating the important role of the DIP0733 in fibrinogen binding.

**Invasion of epithelial cells**

Fibronectin is used as a surface receptor by different pathogenic bacteria, but also triggers internalization of various species, e.g. *Staphylococcus aureus* or *Streptococcus pyogenes* (see Hauck & Ohlsen, 2006; Schwarz-Linek *et al.*, 2006 for reviews). The underlying mechanisms of internalization of *Cor. diphtheriae* by epithelial cells are currently not clear; however, a positive influence of DIP0733 on internalization was proposed based on studies using isolated proteins and antibodies directed against the protein (Sabbadini *et al.*, 2012). When the internalization of wild-type and DIP0733 mutant strain was determined using gentamicin protection assays, the mutant showed a significantly decreased internalization by HeLa cells. Invasion rates of $0.19 \pm 0.03$ % were reached for the wild-type CDC-E8392 and $0.03 \pm 0.01$ % for DIP0733 mutant strain CAM-1. Similar results were obtained for HEp-2 cells (data not shown). As in the case of adhesion assays, transformation of wild-type or strain CAM-1 with vector pXMJ19 (empty vector control) had no influence on invasion rate ($0.16 \pm 0.07$ % and $0.04 \pm 0.03$ %, respectively). In contrast, transformation with DIP0733 expression plasmid pXMJ19-DIP0733 resulted in increased invasion rates, which exceeded wild-type rates by a factor of about three with $0.57 \pm 0.11$ % and $0.48 \pm 0.15$ %, respectively (Fig. 4b). The results obtained support a direct functional role of the DIP0733 protein in respect to the internalization of *Cor. diphtheriae* by epithelial cells.

**Interaction with macrophages**

Previous studies with isolated DIP0733 suggested an influence of the protein not only on the interaction of *Cor. diphtheriae* with extracellular matrix and blood plasma proteins or with epithelial cells but also with macrophages. When the intracellular number of wild-type CDC-E8392 and DIP0733 mutant strain CAM-1 was determined after uptake by THP1 macrophage cells, a clear detrimental effect of the mutation was observed. Two hours after infection, an internalization rate of $1.65 \pm 0.48$ % was determined for wild-type strain CDC-E8392, while strain CAM-1 reached only $0.69 \pm 0.19$ %. Transformation of wild-type or strain CAM-1 with vector pXMJ19 (empty vector control) had no influence on survival or replication within the macrophages ($1.52 \pm 0.66$ % and $1.07 \pm 0.06$ %, respectively), while transformation with DIP0733 expression plasmid pXMJ19-DIP0733 resulted in increased numbers of bacteria, which exceeded wild-type rates by a factor of about two with $2.92 \pm 0.82$ % and $1.69 \pm 0.57$ %, respectively. The number of c.f.u. decreased over time for all strains; however, after 8 and 20 h of infection viable bacteria were still detectable. Again, deletion of DIP0733 decreased and overexpression increased the number of c.f.u. within the macrophage (Fig. 6). In summary, the data obtained indicate that the protein has a beneficial effect on the establishment of *Cor. diphtheriae* within macrophages.

**DISCUSSION**

In the study presented here, we investigated the role of DIP0733 as virulence factor in *Cor. diphtheriae*. The corresponding gene is widely distributed among pathogenic and non-pathogenic corynebacteria, but also found expressed in *Cor. diphtheriae* by epithelial cells.

**Fig. 6.** Interaction of *Cor. diphtheriae* with macrophages. Human THP-1 cells were differentiated into macrophage-like cells using PMA (phorbol 12-myristate 13-acetate) and infected at an m.o.i. of 10 with *Cor. diphtheriae* CDC-E8392 and DIP0733 mutant CAM-1. Intracellular survival (a) 2, (b) 8 and (c) 20 h after infection expressed as a percentage of bacteria used for infection.
in other members of the Actinobacteria. This distribution implicates DIP0733 has other physiological functions besides its role as virulence factor in pathogenic corynebacteria such as Cor. diphtheriae or the closely related Cor. ulcerans.

As a first approach, an invertebrate model system was applied to address the influence of DIP0733 on host interaction. Compared to the wild-type CDC-E8392, DIP0733 mutant CAM-1 exhibited significant attenuation in nematode colonization, proliferation inside the worms and killing of the host. The effect of DIP0733 on host colonization was further investigated and confirmed by experiments using epithelial cells. Adherence and internalization assays revealed decreasing numbers of c.f.u.s of the mutant strain compared to the wild-type. In addition, complementation and overexpression approaches were successful and resulted in strains with increased adhesion and internalization rates implicating a direct role of DIP0733 in these processes. Furthermore, our findings support the idea that invasion of epithelial cells by Cor. diphtheriae represents an important step of infection and that it is advantageous for Cor. diphtheriae to maintain an intracellular location.

At a biochemical level, we observed that DIP0733 deletion affected collagen and fibronectin binding; however, no complete loss was observed but a decrease to about 50% was observed. Many pathogens colonize human tissues or evade immune mechanisms of bacterial clearance exploiting extracellular matrix and/or plasma proteins to colonize human tissues or to evade immune mechanisms for clearance of bacteria (Simpson-Louredo et al., 2014). Sabbadini et al. (2010) suggested that the conversion of fibrinogen to fibrin may be connected to pseudomembrane formation, since differences in the abilities to bind and convert fibrinogen may partially explain differences in the extent of pseudomembrane formation during diphtheria. Obviously, DIP0733 is involved in these processes, but is not exclusively responsible for binding.

Interestingly, Cor. diphtheriae also exhibit strategies to survive within phagocytic cells independent of the tox gene, since also non-toxigenic strains can survive for a considerable time within macrophages (dos Santos et al., 2010). As shown by Sabbadini et al. (2012) and in this study, DIP0733 is involved in processes to avoid and/or delay host defense mechanisms as well as to shield itself from extracellular antibiotics and efficiently persist, disseminate and infect deep tissues. Since phagocytosis by macrophages is independent of bacterial adhesion, it is likely that DIP0733 influences the survival of Cor. diphtheriae within macrophages directly. A putative mechanism in this respect might be the induction of apoptosis, which was indicated by in vitro experiments using isolated DIP0733 protein (Sabbadini et al., 2012).

In summary, DIP0733 seems to be a multi-functional protein with an important role in pathogenicity of Cor. diphtheriae. Unfortunately, a detailed molecular analysis is complicated by a completely lacking annotation of functional domains within the protein (Sabbadini et al., 2012).

Only seven transmembrane helices were identified and also these make handling of the protein more difficult. Future work might therefore rely on extensive mutagenesis studies including truncated DIP0733 forms.

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