Colonization of human epithelial cell lines by *Corynebacterium ulcerans* from human and animal sources

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INTRODUCTION

*Corynebacterium ulcerans* is an emerging pathogen transmitted by a zoonotic pathway to humans. Despite rising numbers of infections and potentially fatal outcomes, data on the colonization of the human host are lacking up to now. In this study, adhesion of two *C. ulcerans* isolates to human epithelial cells, invasion of host cells and the function of two putative virulence factors with respect to these processes were investigated. *C. ulcerans* strains BR-AD22 and 809 were able to adhere to Detroit562 and HeLa cells, and invade these epithelial cell lines with a rate comparable to other pathogens as shown by scanning electron microscopy, fluorescence microscopy and replication assays. Infection led to detrimental effects on the cells as deduced from measurements of transepithelial resistance. Mutant strains of putative virulence factors phospholipase D and DIP0733 homologue CULC22_00609 generated in this study showed no influence on colonization under the experimental conditions tested. The data presented here indicate a high infectious potential of this emerging pathogen.

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BR-AD22, isolated from an asymptomatic dog, and 809, isolated from an 80-year-old woman with fatal pulmonary infection (Mattos-Guaraldi et al., 2008; Dias et al., 2010; Trost et al., 2011). Based on these genome sequences and comparative genomics approaches, a number of putative virulence factors were annotated; however, functional data were scarce. Up to now, data on fibrinogen, fibronectin and collagen binding, antimicrobial profiles, and arthritogenic potential of isolates have been published (Dias et al., 2010; Simpson-Louredo et al., 2011). The function of two putative virulence factors in these initial processes of infection. Bacterial strains, cell lines and plasmids used in this study are listed in Table 1.

**METHODS**

**Bacterial strains and growth.** Non-toxigic C. ulcerans strains BR-AD22 and 809 were grown in Heart Infusion (HI) broth at 37 °C, *Escherichia coli* DH5a/MCR and *Salmonella enterica* serovar Typhimurium NCTC 12023 were grown in Luria Broth (LB) (Sambrook et al., 1989) at 37 °C. If appropriate, kanamycin (60 μg ml⁻¹ for *E. coli*; 50 μg ml⁻¹ for *C. ulcerans*) or chloramphenicol (25 μg ml⁻¹) was added.

**Table 1.** Strains, cell lines and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain, cell line or plasmid</th>
<th>Description/genotype</th>
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<tr>
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<tr>
<td>BR-AD22</td>
<td>Isolated from an asymptomatic dog, non-toxigic (<em>tox</em>⁻)</td>
<td>Mattos-Guaraldi et al. (2008)</td>
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<tr>
<td>809</td>
<td>Isolated from an 80-year-old woman with fatal pulmonary infection, non-toxigic (<em>tox</em>⁻)</td>
<td>Dias et al. (2010)</td>
</tr>
<tr>
<td>ELHA1</td>
<td>BR-AD22 pld : : pK18mob-stdlib, PLD-deficient mutant</td>
<td>Ott et al. (2012)</td>
</tr>
<tr>
<td>ELHA3</td>
<td>BR-AD22 CULC22_00609 : : pK18mob-CULC22_00609'</td>
<td>This study</td>
</tr>
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<td><strong>E. coli</strong></td>
<td></td>
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<tr>
<td>DH5a/MCR</td>
<td>endA1 supE44 thi-1 λ⁻ recA1 gyrA96 relA1 deoR Δ(lacZYA–argF) U196 680 ΔlaczAM15 mcrA Δ(mmrhsdRMS mcrBC)</td>
<td>Grant et al. (1990)</td>
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<td><strong>S. Typhimurium</strong></td>
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<td></td>
</tr>
<tr>
<td>NCTC 12023</td>
<td>WT identical to ATCC 14028</td>
<td>National Collection of Type Cultures (Colindale, UK)</td>
</tr>
<tr>
<td><strong>Cell lines</strong></td>
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<td></td>
</tr>
<tr>
<td>Detroit562</td>
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<td>Peterson et al. (1968)</td>
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<tr>
<td>HeLa</td>
<td>Human cervical carcinoma cells</td>
<td>Gey et al. (1952); Scherer et al. (1953)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pK18mob</td>
<td>ori pUC mob, KmR</td>
<td>Schäfer et al. (1994)</td>
</tr>
<tr>
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<td>pK18mob carrying a 540 bp internal fragment of CULC22_00609 for gene disruption</td>
<td>This study</td>
</tr>
<tr>
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</tr>
<tr>
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<td>This study</td>
</tr>
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<td>pXM19-pld</td>
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<td>This study</td>
</tr>
<tr>
<td>pEPR1-p45gfp</td>
<td>P45 gfpuv rep per T1 T2, KmR</td>
<td>Knoppova et al. (2007)</td>
</tr>
</tbody>
</table>
of the plasmid into the chromosome of C. ulcerans and consequently the disruption of the corresponding genes was confirmed by Southern blotting using specific DNA probes (data not shown).

For overexpression of pld and CULC22_00609 in C. ulcerans BR-AD22, the complete genes were amplified by PCR using chromosomal DNA of strain BR-AD22 as template and the following primers: OE- pld-Pst-I-as (5’-CGCGCTGAGTGTAGAGGGAATCACGATTG-3’)/OE- pld-EcoR-I-as (5’-CGCGAAATTCTGGCGGTGTCTAATCTAC-3’) for amplification of the pld gene and OE-06069-Xbal-as (5’-CCGG-TCTAGAGTGTAGGTAATGGCAGC-3’)/OE-06069-Xmal-as (5’-CGGGCCCCGGGGCTAGGAGTGTATGTAAGC-3’) for amplification of the CULC22_00609 gene. Using the restriction sites introduced via the PCR primers (shown in italic), the DNA fragments were ligated to likewise restricted and dephosphorylated pXMJ19 DNA (Jakoby et al., 1999). The resulting plasmids pXMJ19-pld and pXMJ19-06069 were amplified in E. coli DH5α MCR and used for electroporation of C. ulcerans BR-AD22 as described above. Positive clones were selected on HI agar containing chloramphenicol. RNA hybridization experiments with specific RNA probes proved increased expression levels of the corresponding genes in the resulting C. ulcerans strains (data not shown).

For fluorescence microscopy, electrocompetent C. ulcerans were transformed with gfp-carrying plasmid PEPR1-p45gfp, which leads to constitutive expression of GFP controlled by the p45 corynephage promoter (Knoppova et al., 2007).

Cell cultures. Detroit562 were cultivated in Dulbecco’s modified Eagle’s medium (DMEM), high glucose with l-glutamine and sodium pyruvate (PAA Laboratories), supplemented with 120 μg penicillin ml⁻¹, 120 μg streptomycin ml⁻¹ and 10 % heat-inactivated FCS in a CO₂ incubator. Cells were passaged at a ratio of 1 : 10 twice per week. HeLa cells were cultured in DMEM, high glucose with l-glutamine (PAA Laboratories) supplemented with 100 μg gentamicin ml⁻¹, 12 μg ciprofloxacin ml⁻¹ and 10 % heat-inactivated FCS in a CO₂ incubator. Cells were passaged at a ratio of 1 : 10 twice per week.

Scanning electron microscopy (SEM). Infected monolayer cultures were first fixed with 0.1 % gluteraldehyde, 2 % paraformaldehyde, 5 % sucrose in 0.2 M sodium cacodylate solution for 1 h at 37 °C. Subsequently, samples were fixed with 0.3 % glutaraldehyde, 3 % paraformaldehyde in 0.2 M sodium cacodylate solution for 1 h at 37 °C. Samples were subsequently dehydrated using a graded series of acetone, critical point dried and gold sputtered and examined using a FEI Quanta 200 scanning electron microscope.

Adhesion and invasion assays. Detroit562 and HeLa cells were seeded in 24-well plates (Nunc) at a density of 1 × 10⁴ at 24 h prior to infection. Bacteria were inoculated to OD₆₀₀ 0.1 from overnight cultures and grown in HI broth to OD₆₀₀ 0.4–0.6. Bacteria were harvested by centrifugation and cell density was adjusted to OD₆₀₀ 0.2. A master mix of the inoculum with m.o.i. 50 was prepared in 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 (HeLa cells) or six (Detroit562 cells) times, dettached with 500 μl trypsin solution (0.12 % trypsin, 0.01 % EDTA in PBS) per well (5 min, 37 °C, 5 % CO₂, 90 % humidity) and lysed with 0.025 % Tween 20 for 5 min at 37 °C. Serial dilutions of the lysates and the inoculi in 1 × PBS were plated on Columbia agar with sheep blood (Oxoid) using an Eddy Jet Version 1.22 (IUL Instruments). After incubation at 37 °C for 2 days, the c.f.u. count was determined. The ratio of bacteria used for infection (number of colonies on inoculum plates) and bacteria in the lysate (number of colonies on the lysate plates) multiplied by 100 gave the adhesion efficiency in percent.

For invasion assays, after 90 min of infection, cells were not detached, but 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 once with PBS, detached, lysed and plated as described above, and the c.f.u. count was determined. The ratio of bacteria used for infection (number of colonies on inoculum plates) and bacteria in the lysate (number of colonies on the lysate plates) multiplied by 100 gave the invasion efficiency in percent.

Immunofluorescence microscopy. Detroit562 and HeLa cells were seeded on round coverslips in 24-well plates at a density of 5 × 10⁴ and after 24 h infected at m.o.i. 50 with GFP-expressing C. ulcerans for 90 min. To analyse adhesion, cells were washed six (Detroit562) or three (HeLa) times and then fixed with 4 % paraformaldehyde in PBS for 20 min at 37 °C and stored in PBS at 4 °C until staining. For analysis of invasion, cells were washed three times after 90 min of infection and then further incubated for 2 h with DMEM containing 100 μg ml⁻¹ gentamicin, then washed again and fixed as described above.

Fixed cells were stained with 30 μl Alexa Fluor 647 Phalloidin (Life Technologies) diluted 1 : 200 in an Image-IT FX signal enhancer (Life Technologies) for 45 min in the dark. Subsequently, cells were washed twice with PBS, dried for 5–10 min at 37 °C and then mounted on glass slides using ProLong Gold Antifade Mountant with DAPI (Life Technologies) and stored at 4 °C. Visualization was carried out on the confocal laser scanning microscope Leica SP5 II and analysed with the LAS software suite.

Measurement of transepithelial resistance. Detroit562 cells were seeded in transwells (12 mm, 0.4 μm, polyester membrane, 12-well plate; Corning Costar) at a density of 1 × 10⁵ cells per well and cultivated in DMEM (high glucose, 10 % FCS, 2 mM glutamine) for 14 days until they built a transepithelial resistance of at least 800 Ω cm⁻². Bacteria were subcultured (OD₆₀₀ 0.1 from overnight cultures) in 20 ml HI broth (C. ulcerans) or LB medium (S. Typhimurium) until OD₆₀₀ 0.4-0.6 was reached, harvested by centrifugation and the pellet was resuspended in 500 μl PBS. An OD₆₀₀ of 5 was adjusted in 500 μl PBS and 100 μl of this suspension was used to infect one well. Measurements of transepithelial resistance of Detroit562 cells after infection were carried out with an EVOM2 voltohmmeter (World Precision Instruments) every 60 min. After 180 min the supernatant of infected Detroit562 cells was removed and the cells were incubated in fresh DMEM overnight to avoid detrimental effects of excessive bacterial growth.

RESULTS

SEM

As a first approach, binding of C. ulcerans to epithelial cells was characterized by SEM. For this purpose, adhesion of strains BR-AD22 and 809 to Detroit562 and HeLa cells was analysed. Independent of the C. ulcerans strain applied and the tested epithelial cell line, a localized, clustered adhesion pattern was found (Fig. 1). As tested by microscopic inspection of the inoculum and determination of c.f.u. before and after washing steps, these clusters were not the result of bacterial aggre- gation (data not shown), but hinted at the presence of a limited number of specific cell surface receptor sites. A similar pattern of adhesion of bacteria to the cell surface was observed for different C. diphtheriae strains (Hirata et al., 2004). In the case of Detroit562 cells, in rare cases an insertion of bacteria in the membrane was observed,
suggesting invasion of bacteria into epithelial cells, which are typically non-phagocytic (Fig. 1c).

Fluorescence microscopy

Fluorescence microscopy was used as an independent method to investigate interaction of *C. ulcerans* strains with epithelial cells. For this purpose, both *C. ulcerans* strains were marked by GFP expression, nuclei were stained with DAPI and the cytoskeleton with Alexa Fluor 647 phalloidin (Fig. 2). Fluorescence microscopy images revealed adhesion of the two strains to the surface of Detroit562 and HeLa cells. Internalization of bacteria in the two cell lines was clearly demonstrated by orthogonal z-stacks. Typical V-shaped bacteria due to snapping division indicated growth both on the surface and inside the epithelial cells.

Quantitative analysis of adhesion and invasion

For a quantitative analysis, adhesion and invasion of *C. ulcerans* BR-AD22 and 809 were analysed by determination of c.f.u. Whilst adhering bacteria were directly counted after 90 min, a gentamicin protection assay was used to analyse the rate of internalization (Fig. 3). The two strains were able to adhere to and invade epithelial cells, although to different degrees. In the case of adhesion to Detroit562 cells, a fivefold higher rate was determined for 809 compared with BR-AD22 (187 + 72 versus 31 + 15 %; Fig. 3a), whilst no differences were observed for adhesion to HeLa cells (179 ± 72 versus 186 ± 76 %; Fig. 3c). In the case of Detroit562 infection with strain 809 and the two HeLa infection approaches, strong growth of bacteria was indicated by c.f.u. exceeding the m.o.i. initially applied.

Invasion rates determined were identical for the two *C. ulcerans* strains in the case of Detroit562 cells (0.4 ± 0.1 for BR-AD22 versus 0.3 ± 0.1 % for 809; Fig. 3b), whilst different invasion rates for were found for HeLa infections. In this case, BR-AD22 showed an invasion rate of 2.2 ± 0.5 compared with 5.8 ± 0.7 % for strain 809 (Fig. 3d).

Transepithelial resistance

Epithelial cells infected with *C. ulcerans* appeared to be damaged sometimes; however, whether this was caused by handling effects or a reaction specific for *C. ulcerans* contact remained unclear. Some pathogens, such as *S. Typhimurium*, can cause severe damage on cell membranes and due to the resulting loss of cell integrity, the transepithelial resistance of monolayers is dramatically reduced (e.g. see Gerlach et al., 2008; Ott et al., 2010). In this study, we used *S. Typhimurium* NCTC 12023 as a positive control to test the influence of *C. ulcerans* BR-AD22 and 809 on transepithelial resistance. Whilst the negative control without bacteria showed an even, increasing transepithelial resistance for 4 h, which later stayed constant up to 21 h, infection of Detroit562 monolayers with *S. Typhimurium* caused a fast breakdown of transepithelial resistance within 2 h. Compared with *S. Typhimurium*, effects on monolayers caused by *C. ulcerans* strains BR-AD22 and 809 were considerably slower. Whilst in the case of infection with BR-AD22 > 11 h was needed for breakdown of transepithelial resistance, in the case of strain 809 a low basal resistance level had already been reached at 4 h (Fig. 4). The strong detrimental effect of 809 might be due to the ribosome-binding protein with similarity to Shiga-like toxin encoded by this strain.

Influence of putative *C. ulcerans* virulence factors

Despite increasing numbers of infections and sometimes fatal cases, virulence factors of *C. ulcerans* are widely uncharacterized. However, based especially on homology to closely related pathogenic corynebacteria, a number of *C. ulcerans* proteins have been annotated as putative virulence factors (Trost et al., 2011). Phospholipase D (PLD) is the most important virulence factor of *Corynebacterium pseudotuberculosis*. This so-called *ovis* toxin is involved in dissemination of this animal pathogen from the initial site of infection to other parts of the body. Only two other bacterial species are known to produce a PLD protein similar to that from *C. pseudotuberculosis*: *Arcanobacterium haemolyticum* and *C. ulcerans* (for comparison, see Table 2). Genomic organization of the *pld* gene is similar in *C. ulcerans* and *C. pseudotuberculosis* with a gene encoding a transposase protein and a *fagC* gene flanking *pld*, whilst the *A. haemolyticum pld* is flanked by genes encoding a hypothetical protein on the one side and three tRNAs on the other.

A BR-AD22 strain lacking PLD was generated and designated ELHA1. Compared with the WT, no significant changes in adhesion (BR-AD22 36.1 ± 15.6 versus ELHA1 46.5 ± 16.8 %) or invasion (BR-AD22 0.09 ± 0.04 versus ELHA1 0.1 ± 0.04 %) rate were observed when Detroit562 cells were infected. Overexpression of the protein in strain BR-AD22 pXMJ19-pld also did not influence the adhesion (29.7 ± 8.8 %) or invasion (0.08 ± 0.04 %) rate. Similar results were obtained with HeLa cells (data not shown).

Another example of a putative virulence factor encoded in the *C. ulcerans* genome is CULC22_00609, a homologue of *C. diphtheriae* DIP0733, which was found recently to be involved in adhesion to and invasion of epithelial cells (Antunes et al., 2015). The corresponding protein comprises 81 % identity to DIP0733 from strain *C. diphtheriae* NCTC 13129. A CULC22_00609 gene disruption was generated in *C. ulcerans* BR-AD22, leading to strain ELHA3, and analysed with respect to adhesion and invasion. Disruption of the DIP0733 homologue CULC22_00609 had no effect with respect to adhesion (BR-AD22 33.0 ± 11.7 versus ELHA3 17.5 ± 7.1 %) to Detroit562 cells as well as with respect to invasion of this cell line (BR-AD22...
**Fig. 1.** SEM of *C. ulcerans* and epithelial cells. Interaction of *C. ulcerans* strains with Detroit562 and HeLa cells viewed by SEM at 90 min post-infection. Infected monolayer cultures were fixed, dehydrated, sputtered with gold and examined using a FEI Quanta 200 scanning electron microscope. (a–h) Detroit562 (a–d) and HeLa (e–h) cells infected with BR-AD22 (a, c, e, g) and 809 (b, d, f, h). Putative invasion of bacteria is marked by a white circle in (c). Scale bars represent 10 μm.
0.07 ± 0.02 % versus ELHA3 0.05 ± 0.02 %). Overexpression also showed no significant effects with an adhesion rate of strain BR-AD22 pXMJ19-00609 of 19.1 ± 8.0 % and an invasion rate of 0.08 ± 0.04 %. Again, with HeLa cells also, no influence of the putative virulence factor was found (data not shown).

**DISCUSSION**

*C. ulcerans* is an emerging pathogen, which is transmitted by a zoonotic pathway to the human host (Meinel et al., 2014, 2015). A wide variety of mammals can be infected and provide a reservoir for the pathogen. In recent years the cases of respiratory diphtheria caused by *C. diphtheriae* were outnumbered by *C. ulcerans* infections in Western Europe (Bonmarin et al., 2009; Wagner et al., 2010; Zakikhany & Efstratiou, 2012). Despite increasing numbers of infections and the occurrence of fatal cases, basic mechanisms of host–pathogen interaction and specific virulence factors of *C. ulcerans* have been widely uncharacterized until now.

The two *C. ulcerans* strains investigated in this study were able to bind to epithelial cells – most likely at specific receptor sites – and multiply during *in vitro* infection. Furthermore, the bacteria were able to invade the cytoplasm of epithelial cells. Compared with adhesion rates found for different *C. diphtheriae* strains (for review, see Ott & Burkovski, 2014) and invasion rates determined (e.g. Ott et al., 2013), *C. ulcerans* rates observed here were at least two- to fivefold higher, indicating the high infectious potential of this pathogen. This is further supported by detrimental effects on epithelial cells as shown by the breakdown of transepithelial resistance of cell monolayers after infection with *C. ulcerans* BR-AD22 and 809. Again, also in this case, *C. ulcerans* showed strong effects, whilst an influence on transepithelial resistance was not observed.
for *C. diphtheriae* strains (Ott et al., 2010). The observed internalization into epithelial cells might be an effective mechanism for immune evasion of *C. ulcerans*, supporting the establishment and progress of infections.

Both strain 809, from a fatal case of pneumonia, and BR-AD22, isolated from an asymptomatic dog, were able to colonize human epithelial cells, which might be explained by a close taxonomic relationship as found by Meinel et al. (2014). Nevertheless, strain- and cell line-specific differences observed hint at the existence of more than one mechanism for adhesion and invasion. In line with this idea, initial sequencing of strains 809 and BR-AD22 (Trost et al., 2011) as well as next-generation sequencing of nine *C. ulcerans* isolates revealed a number of different virulence factors which might influence colonization by different isolates of this pathogen. Proteins found included PLD, neuraminidase H, endoglycosidase E, SpaDEF-type adhesive pili, diphtheria toxin, a ribosome-binding protein with similarity to Shiga-like toxin, a haemagglutinin protein, a Fic toxin homologue and a RhuM homologue (Trost et al., 2011; Meinel et al., 2014, 2015; Antunes et al., 2015).

Two putative virulence factors of *C. ulcerans* were tested in this study: the DIP0733 homologue CULC22_00609 and PLD. In *C. diphtheriae* CDC-E8392, DIP0733 is important for adhesion and invasion, and a mutation of the corresponding gene influenced these processes negatively (Antunes et al., 2015). In *C. ulcerans*, an effect on adhesion and invasion by CULC22_00609, the corresponding homologue of DIP0733, was not observed under the experimental conditions tested. The reason for this result is unclear. Possible explanations might be species- and strain-specific differences with respect to adhesion mechanisms, as also observed for 809 and BR-AD22 in this study. However, as we were not able to generate a CULC22_00609 deletion – or any other kind of mutation – in the strain 809 background, this hypothesis could not be verified.

Whilst studies on the influence of PLD on colonization of epithelial cell lines are lacking for *C. pseudotuberculosis*, interaction of a PLD mutant with HeLa cells was tested.

![Fig. 3. Quantitative analysis of *C. ulcerans* adhesion and invasion rates to epithelial cells. Epithelial cells were infected with *C. ulcerans* at m.o.i. 50 for 90 min. (a–d) For analysis of adhesion rates (a, c), the cells were washed six times, detached, lysed and dilutions of the lysate were plated on agar plates to determine the number of attached c.f.u.; for analysis of intracellular c.f.u. (b, d), cells were further incubated for 120 min with medium containing 100 µg gentamicin ml⁻¹ to kill extracellular bacteria, then washed, detached, lysed and plated on agar plates to recover intracellular c.f.u.. (a, b) Detroit562 and (c, d) HeLa. Data shown are mean±sd values of at least three independent biological replicates.](image-url)
In summary, the emerging pathogen *C. ulcerans* includes a broad and varying set of virulence factors (Trost et al., 2011). Furthermore, by acquisition of new genes, this repertoire can change quickly (Meinel et al., 2014), supporting the necessity of constant surveillance of this pathogen (Both et al., 2015). The contribution of putative virulence proteins identified so far and of other components of the cell, like glycolipids, which play an important role in virulence of *C. diptheriae*, *Mycobacterium tuberculosis* and *Rhodococcus equi* (Indrigo et al., 2003; Axelrod et al., 2008; Moreira et al., 2008; Sydor et al., 2013), has to be elucidated in the future at the molecular level by genetic experiments with defined mutants.

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**REFERENCES**


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**Table 2.** Sequence homology of *pld* genes and corresponding PLD proteins of *C. ulcerans*, *C. pseudotuberculosis* and *A. haemolyticum* compared with CULC22_00038, the *pld* gene of *C. ulcerans* BR-AD22

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nucleotide sequence identity (%)</th>
<th>Amino acid sequence identity (%)</th>
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<tr>
<td><em>C. ulcerans</em> 809</td>
<td>97</td>
<td>97</td>
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<tr>
<td><em>C. pseudotuberculosis</em> FRC41</td>
<td>81</td>
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<tr>
<td><em>A. haemolyticum</em> DSM 20595</td>
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Fig. 4. Influence of *C. ulcerans* on transepithelial resistance. Polarized Detroit562 monolayers were grown on transwells and infected with *C. ulcerans* strains (■) BR-AD22 and (▲) 809. For control, cells were incubated without (△) bacteria and (□) *S. Typhimurium*. Experiments were carried out independently at least twice and typical results are shown.


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