THE CYTOLETHAL DISTENDING TOXIN-IV cdt CODING REGION IN AN AVIAN PATHOGENIC ESCHERICHIA COLI (APEC) STRAIN SHOWS INSTABILITY AND IRREGULAR EXCISION PATTERN

ISTVÁN TÓTH¹* and GYÖRGY SCHNEIDER²

¹Institute for Veterinary Medical Research, Agricultural Research Centre, Hungarian Academy of Sciences, Budapest, Hungary
²Institute of Medical Microbiology and Immunology, University of Pécs, Pécs, Hungary

(Received: 1 June 2015; accepted: 2 August 2015)

Cytolethal distending toxins (CDT) represent an emerging toxin family, widely distributed among pathogenic bacteria. The cdtABC genes in E. coli are either part of the genome of prophages, plasmid or pathogenicity island. In order to investigate the stability and the transfer potential of cdt-IV genes cdtB gene was replaced by chloramphenicol (Cm) resistance encoding cat gene in the avian pathogenic E. coli (APEC) strain E250. After consecutive passages in non-selective medium at 37 °C 7.6% (219/2900) of the investigated colonies of E250:cat strain became Cm-sensitive (Cm S). To reveal deletion mechanism 177 CmS colonies were investigated for presence of cdtA, cdtC and cdtC associated gene by PCR. One hundred and sixteen colonies of the Cm S colonies (65.5%) showed partial or complete deletion in the cdt-IV region. Progressive loss of the upstream genes of the cdt cluster in E250 compared to other CDT-IV producing APEC strains and the fact that all the potential deletion patterns were identified, suggests the presence of an unstable hitherto unknown genomic region. The failure of in vitro transfer of cdt genes into a porcine EPEC E. coli strain suggests that the deletion of cdt-IV flanking genes alone do not promote the spread of cdt-IV.

Keywords: cytolethal distending toxin (CDT), Escherichia coli, locus instability

*Corresponding author; E-mail: tothi@vmri.hu
Introduction

A common feature of pathogenic *E. coli* is that their virulence genes are associated with mobile genetic elements. Plasmids, transposons, bacteriophages, pathogenicity islands (PAIs) are the major effectors of horizontal gene transfer [1], which plays a pivotal role in the evolution of pathogenic bacteria [2]. During this process a virulence associated gene or a serial of virulence associated genes can be transferred into non-pathogenic strains and convert them to pathogenic. Acquired genes can be deleted and by this the new feature is lost, while on the other hand a homing process can occur that results the fixation of the new element in the hosts’ chromosome [3]. Traces of this acquisition and the subsequent homing process can be revealed by comparative genomic analysis where hot spots of the integration show a typical mosaic like feature. Presence of partially deleted or mutated bacteriophage and IS element sequences at these sites is a clear indication that bacteriophages can also actively take part in the horizontal transfer of virulence associated genes [3]. Classical and newer examples support the role of bacteriophages in the spread of toxin genes of *Corynebacterium diphtheriae* [4], and the *stx* genes of enterohaemorrhagic *E. coli* (EHEC) O157:H7 strains [5], respectively [6].

Cytolien distending toxin (CDT) – a newly added member of bacterial protein toxins. CDT is a tripartite AB₂ type holotoxin, where CdtB is the active subunit that causes DNA damage in the target cells leading to a characteristic distended morphology and cell cycle arrest in a broad range of mammalian cell lines. CDTs are quite disseminated in several species and so far in *E. coli* five genotypes were identified (reviewed by Tóth and Sváb) [7]. In all cases the *cdtABC* operons were either part of the genome of an inducible converting lambdoid prophage [8], a defective P-2 like prophage [9], a large plasmid [10], as part of a PAI [11] or framed with lambdoid phage genes [12].

Recently as a novel virulence factor CDT-IV production was observed among extraintestinal *E. coli* (ExPEC) isolates of poultry origin [13]. Investigation revealed that *cdt-IV* operon of the avian pathogenic *E. coli* (APEC) strains compared to the structure of the reference *cdt-IV* ExPEC 28C strain contains an altered and still unknown upstream region [12]. That is why in the present study we have carried out a functional analysis with the APEC strain E250 in order to reveal its stability and possible transferability. For this purpose the *cdtB-IV* E250 gene was replaced with a chloramphenicol (Cm) resistance cat gene and loss of the antibiotic resistance was detected by contra-selection. Cm sensitive (S) clones were tested for the presence and by this the affection of flanking regions around the cat cassette.
Materials and Methods

Bacterial strains, plasmids and growth conditions

The *E. coli* strains and plasmids used in the study are listed in Table I. Bacteria were grown in Lysogenic broth (LB) or on LB agar at 37 °C [14]. In the mobilisation experiments, selection was performed on LB agar plates containing chloramphenicol (20 μg/ml) and tetracycline (10 μg/ml). Genetic manipulations with plasmid pSG704 were carried out in the *E. coli* strain SY327 that contains the pir gene (lysogenized with λpir phage), which maintains replicability of the otherwise suicide plasmid. For conjugation the construct was first transformed into the *E. coli* SM10 lambda pir strain that besides possessing the *pir* gene also contains a chromosomally integrated RP4-2 plasmid. That latter one supplies the transacting factors (*tra* genes) necessary to mobilize pSG704 by conjugation [15].

Polymerase chain reaction

The PCR primers used for (i) comparison the genomic organisations of the upstream regions of *cdt* loci between 28C [16] reference strain and APEC E250 [12]; (ii) the cloning steps of labelling, and (iii) screening for deletions after labelling are compiled in Table II.

### Table I. Bacterium strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E250</td>
<td>Avian pathogenic <em>E. coli</em> O115 strain, producing CDT-IV, tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[12]</td>
</tr>
<tr>
<td>28C</td>
<td>Porcine extraintestinal pathogenic <em>E. coli</em> strain of serogroup O75, producing CDT-IV</td>
<td>[16]</td>
</tr>
<tr>
<td>1390</td>
<td>Porcine EPEC strain of serogroup O45, Nal&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[18]</td>
</tr>
<tr>
<td>SY327</td>
<td>F-araD D(lac pro) argE(Am) recA56, rif&lt;sup&gt;R&lt;/sup&gt;, nalA, Pir&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[17]</td>
</tr>
<tr>
<td>SM10 Lambda pir</td>
<td>thi1, thr1, leuB6, supE44, tonA21, lacY1, recA-::RP4-2-TC::Mu, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[18]</td>
</tr>
</tbody>
</table>

Plasmids

- pSG_CDT_M: Recombinant plasmid harbouring the detoxified, *cat* labelled *cdt*-IV operon

---

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene/product</th>
<th>Nucleotid sequence (5‘-3’)</th>
<th>Position in 28C*</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf3-F</td>
<td>orf3 (tail fiber)/cdtA-IV</td>
<td>TATTGCGATATAGCGCGGGGC CTTGCCCTGGAATTCCGACT</td>
<td>3443-3462</td>
<td>2179</td>
<td>This study</td>
</tr>
<tr>
<td>orf4-F</td>
<td>orf4/cdtA-IV</td>
<td>TGTACAGGCATTGATCGGCGG</td>
<td>3873-3892</td>
<td>1749</td>
<td>This study</td>
</tr>
<tr>
<td>orf5-F</td>
<td>orf5/cdtA-IV</td>
<td>ATTCCGGGAGACATCACCAC</td>
<td>4023-4042</td>
<td>1599</td>
<td>This study</td>
</tr>
<tr>
<td>orf6-F</td>
<td>orf6/cdtA-IV</td>
<td>AAATGTCCGGCATTGCAAG</td>
<td>4850-4869</td>
<td>772</td>
<td>This study</td>
</tr>
<tr>
<td>cdtA-IV-s1</td>
<td>cdtA-IV</td>
<td>TGATTCACCTTCGCCACCGGG</td>
<td>5567-5587</td>
<td>558</td>
<td>This study</td>
</tr>
<tr>
<td>rorf1-as1</td>
<td>cdtC-IV/orf1 (protease)</td>
<td>GAGCTGTGCAAATCAAGTCG GAGAAAATCCCGAAAAGACG</td>
<td>7418-7437</td>
<td>632</td>
<td>[12]</td>
</tr>
<tr>
<td>rorf1-as1</td>
<td>rorf1 (protease)</td>
<td>TGACAATCCCCGTGAGAAGACCGCCGATCGATGGAACAC</td>
<td>8163-8186</td>
<td>401</td>
<td>[12]</td>
</tr>
<tr>
<td>CDTE250_44fw(Xhol)</td>
<td>cdtA</td>
<td>TTTCTCAGAGCTGGTTGTGATCGCATGGGATC</td>
<td>5545-5564</td>
<td>648</td>
<td>This study</td>
</tr>
<tr>
<td>CDTE250_713rev(SacI)</td>
<td>cdtA</td>
<td>TTTAGCTCCTCATCCTCCATCCATCCCCATCATG</td>
<td>6214-6193</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDTE250_1501fw(Kpnl)</td>
<td>cdtC (part of cdtB)</td>
<td>TTTGCTACGCTTCTCATCCCGGAAGGAGG</td>
<td>7002-7022</td>
<td>597</td>
<td>This study</td>
</tr>
<tr>
<td>CDTE250_2100rev(Xhol)</td>
<td>cdtC (part of cdtB)</td>
<td>TTTCTCAGGAGATCGTCATGTAATGGAGACATATTGCC</td>
<td>7599-7575</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*GenBank accession: AY578329
In vitro 

Introduction of a chloramphenicol resistance marker, mobilisation genes, an origin of replication, and an origin of transfer into cdt-IV locus of the avian pathogenic E. coli strain E250 was accomplished with the help of plasmid pSG704 [15] as it is depicted in Figure 1, pSG704 is a chloramphenicol-resistant derivative of the conjugative suicide vector pGP704 [15]. The suicide plasmid pSG704 was linearised by digesting it with the restriction enzymes SacI and KpnI. Single recognition sites of these enzymes are localised in the multi cloning site (MCS) of pSG704. PCR fragments (Table II) that correspond to cdtA (CDTE250_44fw(XhoI) – CDTE250_713rev(SacI)) and cdtC (CDTE250_1501fw

Figure 1. Labelling of the cdt locus of the avian pathogen Escherichia coli strain E250 is based on the suicide plasmid pSG704. 
A) pSG704 was linearised with the enzymes SacI and KpnI. 
B) Primers (1–4) with the proper restriction enzyme recognition sites were used to amplify cdtA and cdtB. 
C) Amplified and digested fragments were ligated into pSG704. 
D) The newly constructed plasmid was conjugated into the wild type strain where homologue recombination has occurred. Transconjugants were selected on chloramphenicol plates
(KpnI) – CDTE250_2100rev(XhoI) were ligated in such manner that they could direct a homologue recombination between the plasmid and the chromosome of the wild type strain. Homologue recombination resulted stable integration of the cat cassette, the mob genes, and the oriV_{R6K} in the cdt locus. This replication origin is only functional in the presence of the bacteriophage lambda π-protein. The construct was transformed into Sm10 lambda pir that served as a donor in conjugation experiments.

The Sm10 lambda pir containing the construct was introduced by conjugation to the avian pathogenic E. coli strain E250 served. Donor and recipient strains were grown separately until late logarithmic growth phase and were then mixed with each other according to the following procedure. Donor and recipient strains were adjusted to a ratio of 3:1, were centrifuged and resuspended in LB medium to a final volume of 0.1 ml. This mixture was spotted on a dry agar plate and incubated at 37 °C for 12 hours. Then the plates were washed with 1 ml of sterile sodium chloride (0.9%) and spread on LB agar plates containing chloramphenicol (20 μg/ml) and tetracycline (10 μg/ml). Colonies grown on selective agar after overnight incubation were investigated with cat and cdt specific primers. The colony with proper genotype was used for investigating the stability of cdt-IV locus and its transfer potential.

Screening the stability of the labelled cdt-IV locus in E250:cat

Bacterial suspension grown from the labelled clone was consecutively passaged for 9, 10, or 11 days, in three independent experiments. It was carried out in non-selective LB broth at 37 °C. Randomly chosen isolated clones from outplated proper dilutions of the last passages were picked up and tested for the loss of chloramphenicol resistance marker. Cm sensitive colonies were tested by PCR for the presence of the cdtA, cdtC and the integrity flanking hypothetical protein encoding gene (rorf1).

Investigation of the transferability of the labelled cdt-IV locus experiments

As potential recipient EPEC strain 1390 [18] was used. In these mating experiments the ratio of donor and recipient cultures was 10, 1 and 0.1, respectively. To isolate recombinant (transductant or transconjugant) chloramphenicol was combined with nalidixic acid (50 μg/ml), and the isolated colonies were tested for colicin production.
Results and Discussion

Exploration of the progressive gene loss in upstream region of \textit{cdt-IV} operon in E250

Earlier the characterisation of \textit{cdtABC-IV} region has revealed that \textit{cdt} genes are framed with lambdoid prophage genes in CDT-IV producing ExPEC strains of different origin. Accordingly the downstream region of the \textit{cdt} locus as \textit{cdtC} was always associated with a putative protease (\textit{rorf1}) gene [12]. In contrast the upstream region of the \textit{cdt} locus showed diversity and deletion was detected in some CDT-IV strains including APEC E250 strain. Present investigation indicated that additionally to orf5 and orf6 the gene encoding a tail fiber (orf3) and orf4 are also missing from the E250 strain (Figure 2).

Labelling the \textit{cdt-IV} operon in APEC E250 strain

Integration an antibiotic resistance marker offered the simplest technique to investigate the stability and possible transferability of the \textit{cdt-IV locus}. Re-
placement the cdtB gene by the suicide plasmid pSG704 (Figure 1) by homolo-
gous recombination resulted the labelling of the already non-functional cdt op-
eron with a chloramphenicol resistance cat gene and also supplied mob genes
promoting genetic transfer by conjugation. A derivative colony of E250 harbour-
ing the cat cassette was veriﬁed by PCR and selected for further experiments.
The used construct is analogous to the one we have successfully used for the
mobilisation of the well characterised pathogenicity island II (PAI II536) in the
uropathogenic E. coli (UPEC) strain 536 [15].

Isolation and characterization of chloramphenicol sensitive mutants
from E250:cat derivative strain

From all the 2900 isolated colonies 7.6% (219) proved to be CmS. One hun-
dred and seventy-seven CmS derivative strains were tested by PCR for the pres-
ence of cdtA, cdtC and the integrity of rorf1 encoding a putative prophage pro-
tease. Altogether 65.5% (116/177) of the CmS strains showed partial or complete
deletion in the investigated region and in 61 strains lost only the Cm cassette.
All the seven potential genotypes were identiﬁed among CmS strains and even
partial gene deletion in rorf1 was observed (Table III). This astonishingly high
variability of the deletion pattern we got is in a sharp contrast with former experi-
ments revealing the excision mechanisms of pathogenicity islands. In the case of
PAI II536 a very precise excision pattern was reported that was directed by flank-
ing direct repeats (DRs) on both side of this PAI. Furthermore the excision of a

Table 3. Diversity of cdt-IV region in chloramphenicol sensitive mutants of E250:cat strain

<table>
<thead>
<tr>
<th>cdtA</th>
<th>Genes</th>
<th>protease 5'</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cdtC/protease 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
<td>24 (13.6)</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>–</td>
<td>4 (2.2)</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>+</td>
<td>4 (2.2)</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>+</td>
<td>25 (14.1)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>–</td>
<td>7 (4)</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>+</td>
<td>39 (22)</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>+</td>
<td>13 (7.3)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>61 (34.5)</td>
</tr>
<tr>
<td>111/177 (62.7)</td>
<td>85/177 (48)</td>
<td>138/177 (80)</td>
<td>177 (100)</td>
</tr>
</tbody>
</table>
mobile genetic element is not always precise as it was demonstrated by the classical work of Middendorf et al. [19] in which the island probing technique was used to reveal alterations in the excision patterns of PAI IV\textsubscript{536} the fourth identified PAI of the UPEC strain 536. Precision of such deletion mechanism depends on several factors and mechanisms [20]. Bacterial or phage related IS elements excisionases, integrases, transposases are major vehicles for horizontal genetransfer and by this genome plasticity [1], which plays a pivotal role in the evolution of pathogenic bacteria [2]. Footprints of such integration processes are the presence of mosaic like features that consist of partially deleted or mutated bacteriophage and IS element sequences [3]. Presence of phage related sequences around the \textit{cdt-IV} locus is more than a hint for the fundamental role of temperate phages in the spread of CDT-IV. On the other hand the upstream region of the \textit{cdt-IV locus} in E250 is still unknown. Presence of one or more IS elements in this region can explain the high rate of instability of the \textit{cdt-IV\textsubscript{E250} locus}.

\textit{Transfer experiments}

Thinking about the possible transferability of the \textit{cdt-IV locus} labelling was performed with pSG704 that carries a mob region responsible for conjugation. Our trials to get any recombinant from the transfer experiments has failed, but being aware that efficacies of earlier trials to mobilize well characterized genomic elements [21, 15] was also very low and in both cases the typical feature of these elements were the precise excision and by this specific circularisation, it is not astonishing that in our case transfer of the deleted \textit{cdt locus} could not be carried out.

In summary, we have revealed the progressive loss of bacteriophage related sequences in the APEC strain E250, present next to the \textit{cdt-IV locus} in 28C CDT-IV reference strain and in several investigated ExPEC strains. Unavailable sequence information and the way-out instability of the \textit{cdt-IV} locus strongly suggest the presence of an integration hot spot at this site of the genome of E250 that also could explain the partial or whole deletion of the \textit{cdt} genes.

\textbf{Acknowledgements}

This work was supported by the Hungarian Scientific Research Fund (OTKA), grant number K 81252. We thank Márta Puruczki for technical assistance.
Conflict of Interest

No conflict of interest.

References


