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

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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 Cm^S 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

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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 diphteriae* [4], and the *stx* genes of enterohaemorrhagic *E. coli* (EHEC) O157:H7 strains [5], respectively [6].

Cytholethal distending toxin (CDT) – a newly added member of bacterial protein toxins. CDT is a tripartite AB_2 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 $cdtB\text{-}IV_{E250}$ 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.

Table I. Bacterium strains and plasmids used in this study

Strains	Description	Reference
E250	Avian pathogenic *E. coli* O115 strain, producing CDT-IV, tet^R	[12]
28C	Porcine extraintestinal pathogenic *E. coli* strain of serogroup O75, producing CDT-IV	[16]
1390	Porcine EPEC strain of serogroup O45, Nal^R	[18]
SY327	F-araD D(lac pro) argE(Am) recA56, rif^R, nalA, Pir+	[17]
SM10 Lambda pir	thi1, thr1, leuB6, supE44, tonA21, lacY1, recA-::RP4-2-TC::Mu, Km^R	[18]
Plasmids		
pSG704	Mob fragment from RP4, oriR6K, MCS from M13	[15]
pSG_CDT_M	Recombinant plasmid harbouring the detoxified, *cat* labelled *cdt-IV* operon	This study

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 (10μg/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 II. PCR primers used in this study

Primer	Target gene/product	Nucleotid sequence (5'-3')	Position in 28C*	Size (bp)	Reference
orf3-F	orf3 (tail fiber)/*cdtA-IV*	TATTGCGATATAGGCGGGGC	3443-3462	2179	This study
cdtA-IV-R		CTTGCCCTGGAATTCCGACT	5622-5603		
orf4-F	orf4/*cdtA-IV*	TGTACAGGCATTGATCGCGG	3873-3892	1749	This study
orf5-F	orf5/*cdtA-IV*	ATTCCGGGAGACATCACCAC	4023-4042	1599	This study
orf6-F	orf6/*cdtA-IV*	AAATGTTCGGGCATGGCAAG	4850-4869	772	This study
cdtA-IV-s1	*cdtA-IV*	TGATTCACCTTCGCCACCGGG	5567-5587	558	This study
cdtA-IV-as1		TGCCGCTCTGACAGGTGGAC	6125-6106		
cdtC-IV-s1		GAGCTGTGCAAATCAAGTCG	7418-7437	632	[12]
rorf1-as2	cdtC-IV/rorf1 (protease)	GAGAAAATCCCGAAAAGACG	8031-8050		
rorf1-s1	rorf1 (protease)	TGACAATCCCCTGTGAGAACTGGC	8163-8186	401	[12]
rorf1-as1		CCGTGGCGTATTCGTATGGAACAC	8564-8541		
CDTE250_44fw(*Xho*l)	*cdtA*	TTTCTCGAGCTGGTTGCTCGCATGGGATC	5545-5564	648	This study
CDTE250_713rev(*Sac*l)		TTTGAGCTCCATACTCCCTCCATTCCATCAG	6214-6193		
CDTE250_1501fw(*Kpn*l)	*cdtC* (part of *cdtB*)	TTTGGTACCGCTTCATTCCCGGAAGAGGAG	7002-7022	597	This study
CDTE250_2100rev(*Xho*l)		TTTCTCGAGGATCGTCAATGGAGACATTATTGCC	7599-7575		

*GenBank accession: AY578329

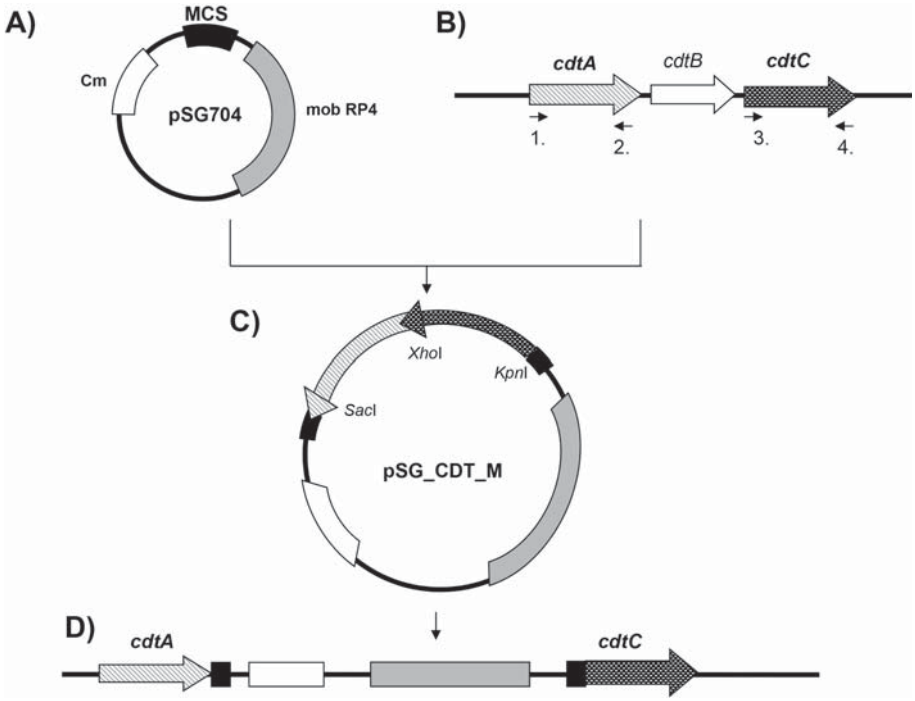

Figure 1. Labelling of the *cdt locus* of the avian pathogen *Escherichia coli* strain E250 is based on the suicide plasmid pSG704.
A) pSG704 was linearised with the enzymes *Sac*I and *Kpn*I.
B) Primers (1–4) with the proper restriction enzyme recognition sites were used to amplify *cdtA* and *cdtB*.
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 occured. Transconjugants were selected on chloramphenicol plates

In vitro *labelling the* cdt-IV *locus*

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 *Sac*I and *Kpn*I. 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(*Xho*I) – CDTE250_713rev(*SacI*)) and *cdtC* (CDTE250_1501fw

(KpnI) – CDTE250_2100rev(*Xho*I)) 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 (10μg/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.

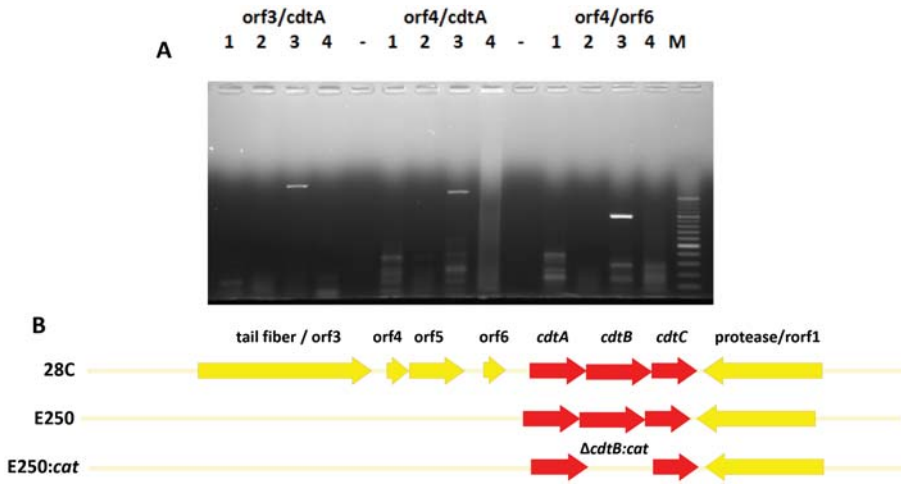

Figure 2. Genomic comparison of *cdtABC-IV* flanking genes in *E. coli* 28C (accession number: AY578329) and APEC E250 strains.
A) The PCR products were separeted by agarose gel electrophoresis and stained with ethidium bromid. The amplicons were loaded in the same order.
Lanes: 1, E250:*cat;* 2, E250; 3, 28C; 4, No DNA; M: 100 bp ladder.
B) The lack of orf3–orf6 flanking genes upstream of *cdtA* and the presence of protease (rorf1) gene downstream of *cdtC* in APEC E250 was demonstrated by PCR

Results and Discussion

Exploration of the progressive gene loss in upstream region of cdt-IV *operon in E250*

Earlier the characterisation of *cdtABC-IV* region has revealed that *cdt* genes are framed with lambdoid prophage genes in CDT-IV producing ExPEC strains of different origin. Accordingly the downstream region of the *cdt locus* as *cdtC* was always associated with a putative protease (rorf1) gene [12]. In contrast the upstream region of the *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 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 *cdt-IV locus*. Re-

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

cdtA	Genes *cdtC*/protease 3'	protease 5'	n (%)
–	–	–	24 (13.6)
–	+	–	4 (2.2)
+	–	–	4 (2.2)
–	–	+	25 (14.1)
+	+	–	7 (4)
+	–	+	39 (22)
–	+	+	13 (7.3)
+	+	+	61 (34.5)
111/177 (62.7)	85/177 (48)	138/177 (80)	177 (100)

placement the *cdtB* gene by the suicide plasmid pSG704 (Figure 1) by homologous recombination resulted the labelling of the already non-functional *cdt* operon with a chloramphenicol resistance *cat* gene and also supplied *mob* genes promoting genetic transfer by conjugation. A derivative colony of E250 harbouring 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 II_{536}) 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 Cm^S. One hundred and seventy-seven Cm^S derivative strains were tested by PCR for the presence of *cdtA*, *cdtC* and the integrity of rorf1 encoding a putative prophage protease. Altogether 65.5% (116/177) of the Cm^S 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 Cm^S 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 experiments revealing the excision mechanisms of pathogenicity islands. In the case of PAI II_{536} a very precise excision pattern was reported that was directed by flanking direct repeats (DRs) on both side of this PAI. Furthermore the excision of a

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_{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 *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 *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 *cdt-*IV_{E250} *locus*.

Transfer experiments

Thinking about the possible transferability of the *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 *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 *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 *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 *cdt* genes.

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Conflict of Interest

No conflict of interest.

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