Liu *et al. Gut Pathog (2016) 8:43*
DOI 10.1186/s13099-016-0125-1

Gut Pathogens

RESEARCH

Open Access

Zonula occludens toxins and their prophages in *Campylobacter* species

Fang Liu, Hoyul Lee, Ruiting Lan and Li Zhang*

Abstract

Background: We previously showed that zonula occludens toxin (Zot) encoded by *Campylobacter concisus zot*^{808T} gene has the potential to initiate inflammatory bowel disease. This Zot protein caused prolonged intestinal epithelial barrier damage, induced intestinal epithelial and macrophage production of tumor necrosis factor-α and enhanced the responses of macrophages to other microbes. In order to understand the potential virulence of Zot proteins in other *Campylobacter* species, in this study we examined their presence, similarities, motifs and prophages.

Methods: The presence of Zot proteins in *Campylobacter* species was examined by searching for the Zot family domain in multiple protein databases. Walker A and Walker B motifs in Zot proteins were identified using protein sequence alignment. A phylogenetic tree based on *Campylobacter zot* genes was constructed using maximum-likelihood method. *Campylobacter* Zot proteins were compared using protein sequence alignment. The *zot*-containing prophages in *Campylobacter* species were identified and compared with known prophage proteins and other viral proteins using protein sequence alignment and protein BLAST.

Results: Twelve Zot proteins were found in nine *Campylobacter* species/subspecies. Among these *Campylobacter* species, three species had two Zot proteins and the remaining six species/subspecies had one Zot protein. Walker A and Walker B motifs and a transmembrane domain were found in all identified *Campylobacter* Zot proteins. The twelve *Campylobacter zot* genes from the nine *Campylobacter* species/subspecies formed two clusters. The $Zot_{CampyType\_1}$ proteins encoded by Cluster 1 *Campylobacter zot* genes showed high similarities to each other. However, $Zot_{CampyType\_2}$ proteins encoded by Cluster 2 *Campylobacter zot* genes were more diverse. Furthermore, the *zot*-containing *Campylobacter* prophages were identified.

Conclusion: This study reports the identification of two types of *Campylobacter* Zot proteins. The high similarities of $Zot_{CampyType\_1}$ proteins suggest that they are likely to have similar virulence. $Zot_{CampyType\_2}$ proteins are less similar to each other and their virulent properties, if any, remain to be examined individually.

Keywords: *Campylobacter concisus*, Zonula occludens toxin (Zot), *Campylobacter*, Prophage

Background

Campylobacter concisus is a Gram-negative spiral shaped motile bacterium [1]. Their growth under both anaerobic and microaerobic conditions is largely determined by the presence of H_2 [2]. This bacterium usually colonizes the human oral cavity [3, 4]. However, it may also colonize the intestinal tract of some individuals and its prevalence in intestinal biopsies has been associated with human inflammatory bowel disease (IBD) [5–9]. Translocation of *C. concisus* from the oral cavity to intestinal tract has been suggested to be a cause of a subgroup of IBD [10]. *Campylobacter concisus* has also been suggested to be involved in diarrheal disease due to the frequent isolation of this bacterium from diarrheal stool samples [8, 11–13].

Campylobacter concisus zonula occludens toxin (Zot) is encoded by the *zot* gene located in CON_phi2 prophage [14, 15]. The *zot* genes in different *C. concisus* strains have polymorphisms [14]. In a recent study, we examined the effects of *C. concisus* Zot encoded by *zot*^{808T} gene on human intestinal epithelial cells and macrophages using cell line

*Correspondence: L.Zhang@unsw.edu.au
School of Biotechnology and Biomolecular Sciences, University of New South Wales, Kensington, Sydney 2052, Australia

models. In that study, we found that *C. concisus* Zot caused prolonged intestinal epithelial barrier damage, induced intestinal epithelial and macrophage production of proinflammatory cytokines such as tumor necrosis factor-α, and enhanced the responses of macrophages to *Escherichia coli* [16]. These data suggest that *C. concisus* Zot may play a role in initiating chronic intestinal inflammatory conditions such as IBD through damaging the intestinal barrier and enhancing the immune responses to luminal microbes.

In addition to *C. concisus*, four additional *Campylobacter* species including *Campylobacter ureolyticus*, *Campylobacter corcagiensis*, *Campylobacter gracilis* and *Campylobacter iguaniorum* were recently reported to possess the *zot* gene [17–21]. The similarities of Zot proteins in different *Campylobacter* species have not been examined. In this study, we examined Zot proteins and their genes in *Campylobacter* species, which revealed two clusters of *Campylobacter zot* genes and two types of *Campylobacter* Zot proteins. Furthermore, we identified the prophages that contain *zot* genes in *Campylobacter* species.

Methods

Examination of the presence of Zot proteins in *Campylobacter* species

The presence of Zot proteins in *Campylobacter* species was examined by searching for proteins in *Campylobacter* species that have the Zot family domain in multiple protein databases including NCBI protein database, InterPro and Pfam [22–24]. The Zot proteins in these databases were annotated based on the presence of Zot family domain.

Examination of the presence of Walker A and Walker B in *Campylobacter* Zot proteins

It is shown in the InterPro database that the Zot family proteins (InterPro entry identity: IPR008900) belong to p-loop containing nucleoside triphosphate hydrolase (p-loop NTPase) superfamily. The proteins of p-loop NTPase superfamily have Walker A and Walker B motifs [25]. In this study, we examined the presence of Walker A and Walker B motifs in *Campylobacter* Zot proteins by protein alignment using Clustal Omega software [26]. The Walker A motif has a sequence of GxxxxGK[S/T], where x is any residue and the Walker B motif has a sequence of hhhh[D/E], where h is a hydrophobic residue [25].

Generation of a phylogenetic tree based on *zot* genes in *Campylobacter* species

To examine the genetic relationship between the *zot* genes in different *Campylobacter* species, the nucleotide sequences of the *zot* genes in *Campylobacter* species identified above were obtained from NCBI genome database. These sequences were used to generate a phylogenetic tree using the maximum likelihood method implemented in molecular evolutionary genetics analysis software version 6.0 [27]. In order to differentiate the *zot* genes in different *Campylobacter* species, the *zot* genes found in different *Campylobacter* species were indicated by the last four digits of their locus tag numbers.

Comparison of *zot*-containing prophages in *Campylobacter* species

To examine whether the *zot* genes in different *Campylobacter* species are carried by prophages similar to that in *C. concisus*, the *zot*-containing prophages in these *Campylobacter* species were identified by examination of the genes adjacent to their *zot* genes. The prophages were defined based on the presence of integrase, hypothetical proteins and attachment sites [28]. The attachment sites were identified by the presence of repetitive sequences located at both ends of the prophages [28].

The proteins in the identified *Campylobacter* prophages in this study were compared with the corresponding proteins in *C. concisus* prophages CON_phi2 and CON_phi3. The comparison was performed using Clustal Omega [26]. Proteins sharing more than 40 % sequence identity were considered to have high similarities and were recorded [29].

Comparison of the proteins of *zot*-containing prophages in *Campylobacter* species with other viral proteins

To examine whether the *zot* containing prophages identified in *Campylobacter* species are similar to previously reported prophages, proteins of *Campylobacter* prophages were compared with viral proteins in the NCBI non-redundant protein sequence database (taxonomy identity for viruses: 10,239) using protein BLAST with default settings [22]. The identified viral proteins with E-values lower than 10 were noted. For prophage proteins which shared sequence similarities with multiple viral proteins, the viral proteins with the lowest E-values were noted. The full length sequences of the identified viral proteins were then aligned with the *Campylobacter* prophage proteins using Clustal Omega [26]. The protein identities were calculated by dividing the number of identical amino acids by the total number of amino acids in proteins from *Campylobacter* prophages. The *Campylobacter* prophage proteins were also compared with viral proteins in the virus pathogen database and analysis resource (ViPR) using BLAST with a cut-off E-value of 10. In addition to *C. concisus*, the *zot* gene was also found in other bacterial species such as *Vibrio cholerae* and *Neisseria meningitis* [30, 31]. The

identities between *Campylobacter* Zot, *V. cholerae* Zot and *N. meningitidis* Zot proteins were also compared in this study.

Prediction of secreted proteins and transmembrane proteins in *Campylobacter* prophages

Secreted proteins in *zot*-containing prophages in different *Campylobacter* species were predicted using SignalP 4.1 and SecretomeP 2.0. The software SignalP 4.1 predicts secreted proteins based on the presence of the signal peptide at the N-terminus [32]. The software SecretomeP 2.0 predicts non-classical (not signal peptide triggered) protein secretion based on analysis of post-translational and localizational aspects of the proteins [33]. Transmembrane proteins were predicted using the software Phobius [34].

Results

The Zot proteins in different *Campylobacter* species and their Walker A and Walker B motifs

Twelve Zot proteins were found in nine *Campylobacter* species/subspecies. Three *Campylobacter* species including *C. concisus*, *C. ureolyticus* and *C. corcagiensis* had two Zot proteins and the remaining six *Campylobacter* species/subspecies including *C. gracilis*, *Campylobacter jejuni* subsp. *doylei*, *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter hyointestinalis* subsp. *hyointestinalis*, *C. hyointestinalis* subsp. *lawsonii* and *C. iguaniorum* had one Zot protein (Table 1).

The Zot family domains were localized at the N-terminal side of the identified *Campylobacter* Zot proteins, prior to the transmembrane domains. The Zot family proteins had p-loop NTPase domains and the entry identity for p-loop NTPase domain was IPR027417 in InterPro database. We identified the Walker A and Walker B motifs in *Campylobacter* Zot proteins, which were at the N-terminal side of the Zot proteins (Fig. 1).

The phylogenetic tree formed based on *zot* genes in *Campylobacter* species

The *Campylobacter zot* genes formed two clusters. Cluster 1 contained three *zot* genes, including *C. concisus zot2276, C. ureolyticus zot3935 and C. corcagiensis zot6485.* Cluster 2 contained nine *Campylobacter zot* genes (Fig. 2).

Comparison of $Zot_{CampyType\_1}$ and $Zot_{CampyType\_2}$ proteins

The Zot proteins encoded by Cluster 1 and Cluster 2 *Campylobacter zot* genes were referred to as $Zot_{CampyType\_1}$ and $Zot_{CampyType\_2}$ proteins respectively. The three $Zot_{CampyType\_1}$ proteins had high similarities; they shared 171 identical amino acids and 77 conservative mutations (Fig. 3). The $Zot_{CampyType\_2}$ proteins were less similar to each other as compared to $Zot_{CampyType\_1}$ proteins; they had 71 identical amino acids and 65 conservative mutations (Fig. 4).

The *zot*-containing prophages in different *Campylobacter* species and their similarities to *C. concisus* prophages CON_phi2 or CON_phi3

We identified the *zot*-containing prophages in different *Campylobacter* species. Each of these prophages began with an integrase and had a number of hypothetical proteins (Fig. 5; Additional file 1). The attachment sites were found (Table 2). These prophages were inserted within either tRNA-Met or tRNA-Ser genes, except for URE_phiZB, which was inserted into tRNA-Leu gene (Table 2). For the prophage in *C. iguaniorum*, two tRNA genes were found after the integrase, suggesting that multiple insertions have occurred.

Table 1 Zot proteins in *Campylobacter* species/subspecies

Campylobacter species/subspecies	Strain	Number of Zot proteins	Locus tag	Source of isolation	Reference
C. concisus	13826	2	CCC13826_2276 CCC13826_0191	Human faeces	Gb0000058[a]
C. ureolyticus	DSM 20703	2	C512_RS0103935 C512_RS0100745	Human amniotic fluid	[35]
C. corcagiensis	CIT045	2	BG71_RS0106485 BG71_RS0104620	Lion-tailed macaques faeces	[36]
C. gracilis	RM3268	1	CAMGR0001_2456	Human oral cavity	Gb0003988[a]
C. jejuni ssp. *doylei*	269.97	1	JJD26997_0348	Human blood	Gb0000076[a]
C. jejuni ssp. *jejuni*	60004	1	CJE11_RS08060	Chicken	SAMN02429007[@]
C. hyointestinalis ssp. *hyointestinalis*	DSM 19053	1	CR67_01870	Porcine intestine	SAMN01176354[@]
C. hyointestinalis ssp. *lawsonii*	CCUG 27631	1	CHL_RS06765	Porcine intestine	[37]
C. iguaniorum	RM11343	1	CIG11343_RS03950	Alpaca faeces	[21]

[a] Genome online database project ID. [@]NCBI Biosample ID

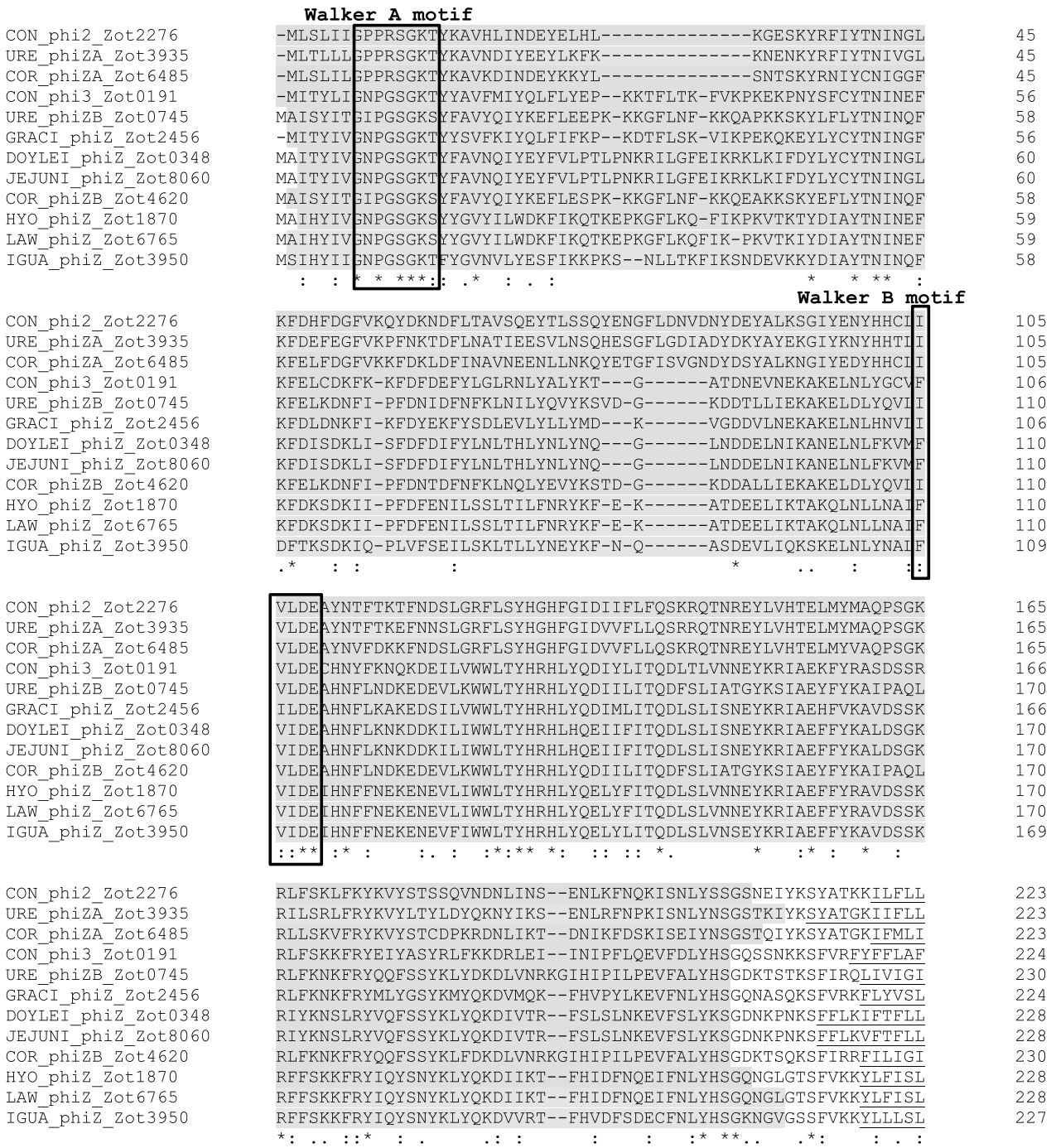


Fig. 1 Walker A and walker B motifs in *Campylobacter* Zot proteins. *Campylobacter* Zot proteins have a transmembrane domain (*underlined*). The amino acids prior to the transmembrane domains constitute the Zot family domains (approximately 1–210, *shaded in grey*) in different *Campylobacter* Zot. The Zot family domains belong to p-loop NTPase superfamily. Walker A and walker B motifs are in the N-terminus of *Campylobacter* Zot proteins. Walker A has a sequence of GxxxxGK[S/T], where x is any residue. Walker B motif has a sequence of hhhh[D/E], where h is a hydrophobic residue [25]

Prophages containing $Zot_{CampyType\_1}$ proteins had high similarities to CON_phi2. Eight proteins in URE_phiZA and nine proteins in COR_phiZA were found to have more than 40 % identities (41-73 %) with proteins in CON_phi2 (Fig. 5a; Additional file 1). Proteins in prophages containing $Zot_{CampyType\_2}$ proteins were more diverse. However,

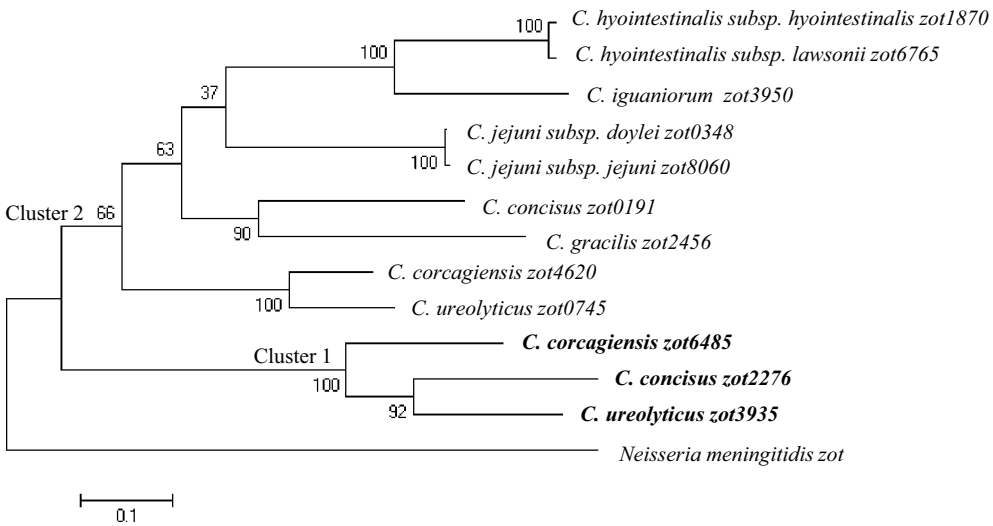


Fig. 2 The phylogenetic tree generated based on *zot* genes in different *Campylobacter* species. Maximum likelihood method was used to generate the phylogenetic tree. *Bootstrap* values were generated from 1000 replicates. Cluster 1 *zot* genes are shown in *bold*. The *zot* gene from *Neisseria meningitides* (strain 69166) was used as the outgroup

```
CON_phi2_Zot2276      MLSLIIGPPRSGKTYKAVHLINDEYELHLKGESKYRFIYTNINGLKFDHFDGFVKQYDKN  60
URE_phiZA_Zot3935     MLTLLLGPPRSGKTYKAVNDIYEEYLKFKKNENKYRFIYTNIVGLKFDEFEGFVKPFNKT  60
COR_phiZA_Zot6485     MLSLILGPPRSGKTYKAVKDINDEYKKYLSNTSKYRNIYCNIGGFKFELFDGFVKKFDKL  60
                      **:*::************. * :**  . .  .*** ** ** *:**: *:**** ::*

CON_phi2_Zot2276      DFLTAVSQEYTLSSQYENGFLDNVDNYDEYALKSGIYENYHHCLIVLDEAYNTFTKTFND  120
URE_phiZA_Zot3935     DFLNATIEESVLNSQHESGFLGDIADYDKYAYEKGIYKNYHHTLIVLDEAYNTFTKEFNN  120
COR_phiZA_Zot6485     DFINAVNEENLLNKQYETGFISVGNDYDSYALKNGIYEDYHHCLIVLDEAYNVFDKKFND  120
                      **:.*. :*  *..*:*.**:    :**.** :.***::*** *********.* * **:

CON_phi2_Zot2276      SLGRFLSYHGHFGIDIIFLFQSKRQTNREYLVHTELMYMAQPSGKRLFSKLFKYKVYSTS  180
URE_phiZA_Zot3935     SLGRFLSYHGHFGIDVVFLLQSRRQTNREYLVHTELMYMAQPSGKRILSRLFRYKVYLTY  180
COR_phiZA_Zot6485     SLGRFLSYHGHFGIDVVFLLQSKRQTNREYLVHTELMYVAQPSGKRLLSKVFRYKVYSTC  180
                      ***************::**:**:***************:*******::*::*:**** *

CON_phi2_Zot2276      SQVNDNLINSENLKFNQKISNLYSSGSNEIYKSYATKKILFLLAFIVFSYVVYKFLEPKH  240
URE_phiZA_Zot3935     LDYQKNYIKSENLRFNPKISNLYNSGSTKIYKSYATGKIIFLLLIIFISYFGYKFLKPKP  240
COR_phiZA_Zot6485     DPKRDNLIKTDNIKFDSKISEIYNSGSTQIYKSYATGKIFMLIVLAIILYFGFKFIGPPK  240
                         ..* *:::*::*: ***::*.***.:******* **::*: : .: *. :**: *

CON_phi2_Zot2276      EPAQSTKQETRFVDLNASD--SKNIKAISNDADKSDINTTIFNDNKIYLRITCFPSGCKF  298
URE_phiZA_Zot3935     AKQETIITDERFKDINRTNQDIKEPQLIQNSDLNLDLNTTIFNDKRTYLKITCYSHFCKF  300
COR_phiZA_Zot6485     LENDKAKKDEFISEIYVSDKN--QTLEISNYKETIKDENLLLNERRIYEKITCFPSSCKF  298
                         :.   :  : ::  ::    :   *.*   . . :. ::*:.: * :***:   ***

CON_phi2_Zot2276      RNYAIDLSLDSFLELLSSSNCHIFLHDKKSGNYIDYFVSCNSEFERVLKGLENSSQRVC-  357
URE_phiZA_Zot3935     RNYSLDLSLNSFLELISSFDCYIFLKDEKSANYADYYLSCPLDFSKVVSNIND-LQEIC-  358
COR_phiZA_Zot6485     RSYSLNLTLDSFLLLLADSKCSIVLTDKKSSNYIDYYVSCPAEFIGFLSKFSGDDNFYKG  358
                      *.*:::*:*:*** *:::. .* *.* *:**.** **::**  :*  .:. :.   :

CON_phi2_Zot2276      --NE-KSPQTDSSSMFPTHK        374
URE_phiZA_Zot3935     --DE-NKGSFNTFSFK----        371
COR_phiZA_Zot6485     SQNENYTKNYNSFDFR----        374
                        :*  . . :: .:
```

Fig. 3 Comparison of $Zot_{CampyType\_1}$ proteins. The protein similarities were compared using Clustal Omega. *Asterisk* indicates identical amino acids (*shaded in red*). *Colon* indicates conservative mutations (*shaded in blue*). *Dot* indicates semi-conservative mutations. Transmembrane domains are *underlined*

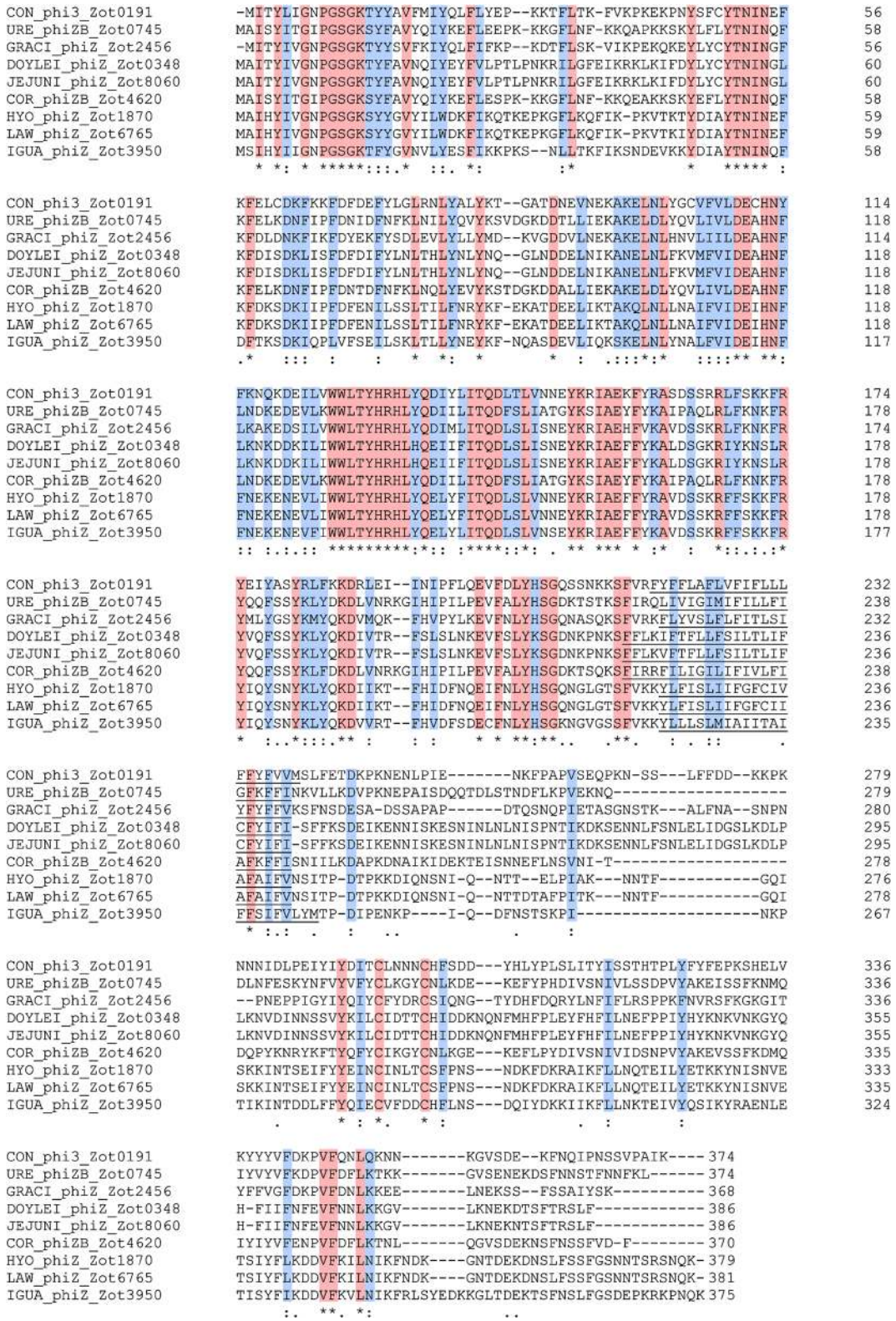
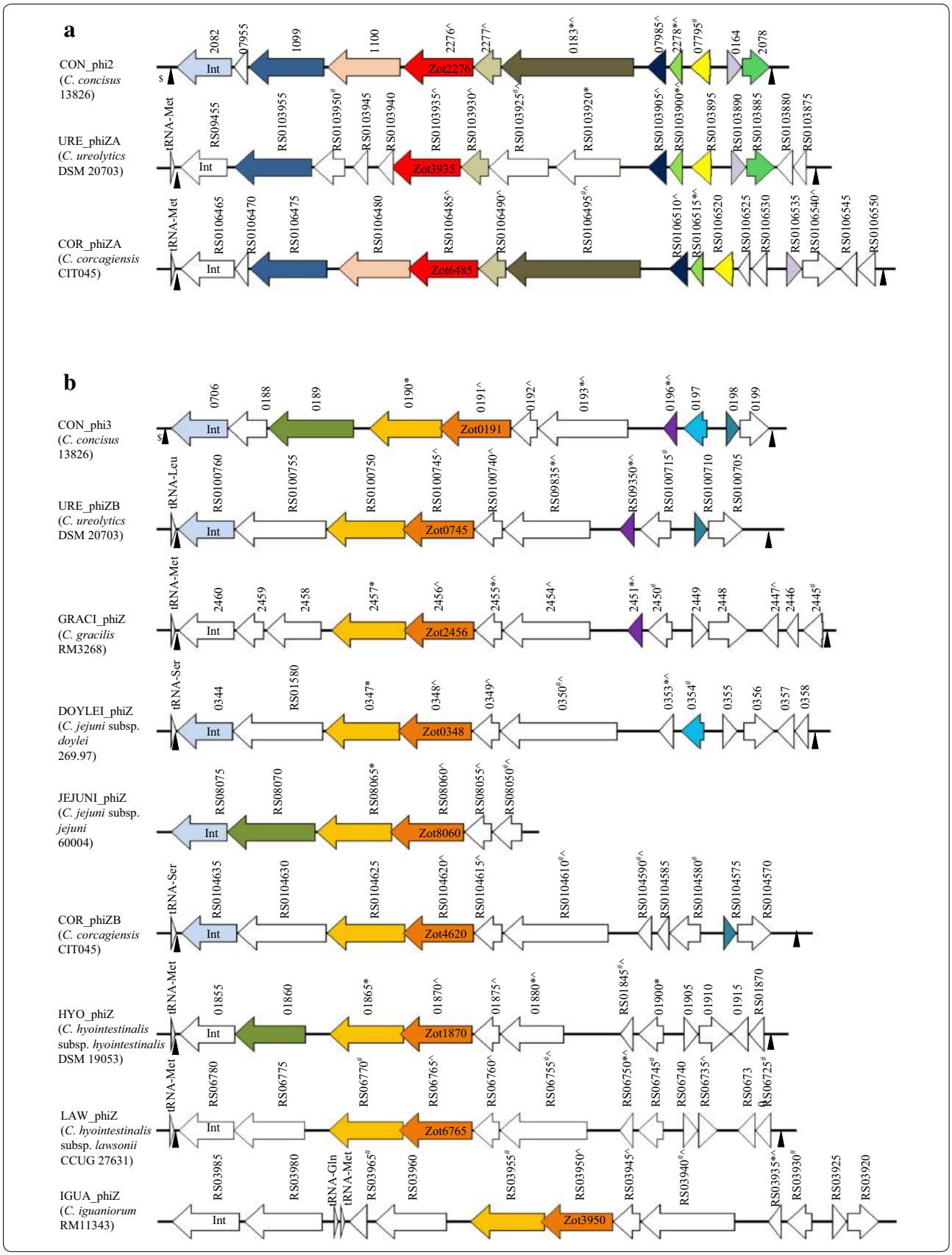


Fig. 4 Comparison of $Zot_{CampyType\_2}$ proteins. The protein similarities were compared using Clustal Omega. *Asterisk* indicates identical amino acids (*shaded in red*). *Colon* indicates conservative mutations (*shaded in blue*). *Dot* indicates semi-conservative mutations. Transmembrane domains are *underlined*


a
CON_phi2 (C. concisus 13826)
URE_phiZA (C. ureolytics DSM 20703)
COR_phiZA (C. corcagiensis CIT045)
b
CON_phi3 (C. concisus 13826)
URE_phiZB (C. ureolytics DSM 20703)
GRACI_phiZ (C. gracilis RM3268)
DOYLEI_phiZ (C. jejuni subsp. doylei 269.97)
JEJUNI_phiZ (C. jejuni subsp. jejuni 60004)
COR_phiZB (C. corcagiensis CIT045)
HYO_phiZ (C. hyointestinalis subsp. hyointestinalis DSM 19053)
LAW_phiZ (C. hyointestinalis subsp. lawsonii CCUG 27631)
IGUA_phiZ (C. iguaniorum RM11343)

(See figrue on previous page.)
Fig. 5 Schematic illustration of protein similarities in *zot*-containing *Campylobacter* prophages. **a** *Campylobacter* prophages containing $Zot_{CampyType\_1}$ proteins. **b** *Campylobacter* prophages containing $Zot_{CampyType\_2}$ proteins. The prophages and their host *Campylobacter* strains (*in bracket*) are listed at the left side of the figure. Proteins with more than 40 % identical amino acids with proteins in CON_phi2 or CON_phi3 were labeled with the same color. The numbers above the proteins are locus tags of the genes in the NCBI database. *Int* indicates integrase. *Asterisk* and *Hashtag* indicate proteins predicted to be secreted via classic secretory pathway or non-classic secretory pathway respectively. *Caret* indicates transmembrane proteins. *Dollar* indicates multiple insertion sites for CON_phi prophages in *C. concisus* 13826 in which only the first attachment site (for CON_phi1) overlapped with tRNA [15]. *Filled triangle* indicates attachment sites

three to five proteins in these prophages had more than 40 % identities with that in CON_phi3 (40–72 %) (Fig. 5b; Additional file 1).

The similarities between proteins in *Campylobacter* prophages and viral proteins

The *zot*-containing *Campylobacter* were compared with known viral proteins in NCBI non-redundant protein sequence database. The proteins within each individual prophage showed low similarities to multiple phage proteins, except for CCC13826_0188 in CON_phi3 that shared 43 % identity with a phage transferase from an uncultured phage (Additional file 2). The Zot proteins in *Campylobacter* prophages had low similarities with the Zot proteins in *V. cholerae* and *N. meningitidis* (15–21 %) (Additional files 3, 4). None of the *Campylobacter* prophage proteins shared significant similarities with viral proteins in ViPR database. These data suggest that the *zot*-containing prophages in *Campylobacter* species are new prophages that have not been previously characterized.

Secreted and transmembrane proteins in *Campylobacter* prophages

Proteins secreted via both classical secretory pathway (with signal peptides) and non-classical secretory pathway (without signal peptides), as well as transmembrane proteins were found in all *Campylobacter* prophages (Fig. 5).

Discussion

In addition to *C. concisus*, a number of other *Campylobacter* species were recently reported to have the *zot* genes [17–21]. In this study, we found that *Campylobacter zot* genes formed two clusters (Fig. 2). Most of the *Campylobacter zot* genes were in Cluster 2, and Cluster 1 contained only three *zot* genes. The three *Campylobacter* species that had Cluster 1 *zot* genes also contained Cluster 2 *zot* genes. The remaining six *Campylobacter* species/subspecies contained Cluster 2 *zot* genes only. These data show that Cluster 2 *zot* gene is more prevalent in *Campylobacter* species as compared to Cluster 1 *zot* genes.

$Zot_{CampyType\_1}$ proteins, which were encoded by Cluster 1 *zot* genes, were highly similar to each other. However they were less similar to $Zot_{CampyType\_2}$ proteins that were encoded by Cluster 2 *Campylobacter zot* genes. The *zot* gene in CON_phi2 prophage in *C. concisus* belongs to $Zot_{CampyType\_1}$. Using cell culture models, we previously showed that $Zot_{CampyType\_1}$ in *C. concisus* encoded by zot^{808T} polymorphism damaged intestinal epithelial barrier by induction of epithelial apoptosis and induced production of proinflammatory cytokines such as TNF-α in HT-29 cells and THP-1 macrophage-like cells, supporting its role as a potential virulence factor [16]. The high similarities between $Zot_{CampyType\_1}$ proteins in the three different *Campylobacter* species suggest that they may have similar effects on human cells. Great variations in protein sequences between $Zot_{CampyType\_1}$ and $Zot_{CampyType\_2}$ proteins as well as within $Zot_{CampyType\_2}$ proteins were observed in this study. Given this, the effects of $Zot_{CampyType\_2}$ proteins on human cells, if any, require to be examined individually.

A transmembrane domain was found in all Zot proteins, showing that Zot proteins are transmembrane proteins. Furthermore, all Zot proteins contained Walker A and Walker B motifs, which are conserved motifs of p-loop NTPase superfamily [25]. P-loop NTPase bind to NTP typically ATP or GTP through the Walker A and B motifs, which are involved in diverse cellular functions [25]. Future studies should be conducted to examine whether *Campylobacter* Zot proteins have NTPase activities.

In this study, we identified a number of *zot*-containing prophages in other *Campylobacter* species in additional to previous reported prophages in *C. concisus* (Fig. 5). These prophages have an integrase, a number of hypothetical proteins and attachment sites (Fig. 5; Table 2; Additional file 1), which satisfy the previously defined criteria for prophages [28]. The proteins in individual *Campylobacter* prophages identified in this study have low similarities with multiple viral proteins, suggesting that they are new prophages that have not being characterized previously (Additional file 2).

Campylobacter Zot proteins had very low similarities to *V. cholerae* Zot and *N. meningitis* Zot proteins (Additional files 3, 4). These data showed that despite having a common name, the amino acid sequences of Zot proteins in different bacterial species vary greatly. Thus, they may not necessarily exhibit the same effects on human cells.

Table 2 The attachment sites of *zot*-containing *Campylobacter* prophages

Prophage	Start[a]	End[a]	Attachment gene sequence[b]	tRNA (locus_tag)
CON_phi1, CON_phi2 and CON_phi3 (*C. concisus* 13826)[c]	1582286	1582311	*TTCAAATCCCTCTCTGTCCGCCA*CCA	tRNA-Ser (CCC13826_RS07905)
	1587508	1587533	TTCAAATCCCTCTCTGTCCGCCACCA	
	1597113	1597138	TTCAAATCCCTCTCTGTCCGCCACCA	
	1606718	1606743	TTCAAATCCCTCTCTGTCCGCCACCA	
	1616160	1616185	TTCAAATCCCTCTCTGTCCGCCACCA	
CON_phi4 (*C. concisus* 13826)	946941	946993	*CTCATAACCCGAAGGTCGGCGGTTCAA ATCCGTCCTCCGCAACCA*AATACCGA	tRNA-Met (CCC13826_RS04780)
	937290	937342	CTCATAACCCGAAGGTCGGCGGTTCAA ATCCGTCCTCCGCAACCAAATACCGA	
URE_phiZA (*C. ureolyticus* DSM 20703)	68067	68122	*ATAACCCGAAGGTCGGAGGTTCAAGTCCT TCCTCTGCAACCA*AATCACCATTTTAC	tRNA-Met (C512_RS0103965)
	57811	57866	ATAACCCGAAGGTCGGAGGTTCAAGTCCT TCCTCTGCAACCAAATCACCATTTTAC	
URE_phiZB (*C. ureolyticus* DSM 20703)	139564	139610	*GTTCAAGTCTCGCTGATCGCACCA*TTA AAGAAAAAATTAAGAATACT	tRNA-Leu (C512_RS0100765)
	130103	130149	GTTCAAGTCTCGCTGATCGCACCATTA AAGAAAAAATTAAGAATACT	
COR_phiZA (*C. corcagiensis* CIT045)	254051	254088	*CGAAGGTCAGGGGTTCAAGTCCCTTCT CTGCAACCA*AA	tRNA-Met (SA94_RS06290)
	265302	265339	CGAAGGTCAGGGGTTCAAGTCCCTTCT CTGCAACCAAA	
COR_phiZB (*C. corcagiensis* CIT045)	257240	257264	*GTTCAAATCCCTCTCTGTCCGCCA*C	tRNA-Ser (SA94_RS04510)
	247319	247343	GTTCAAATCCCTCTCTGTCCGCCAC	
GRACI_phiZ (*C. gracilis* RM3268)	112826	112871	*CTCATAACCCGAAGGTCGGTGGTTCAA ATCCACCCTCTGCAACCAA*	tRNA-Met (CAMGR0001_2931)
	102518	102563	CTCATAACCCGAAGGTCGGTGGTTCAA ATCCACCCTCTGCAACCAA	
DOYLEI_phiZ (*C. jejuni* subsp. *doylei* 269.97)	303215	303241	*AGGGTTCAAATCCCTCTCTGTCCGCCA*	tRNA-Ser (JJD26997_RS01570)
	313165	313191	AGGGTTCAAATCCCTCTCTGTCCGCCA	
HYO_phiZ (*C. hyointestinalis* subsp. *hyointestinalis* DSM 19053)	360409	360446	*CTCATAACCCGAAGGTCGGAGGTTCAA GTCCTTCTCTC*	tRNA-Met (CR67_RS01810)
	369716	369753	CTCATAACCCGAAGGTCGGAGGTTCAA GTCCTTCTCTC	
HYO_phiZ (*C. hyointestinalis* subsp. *lawsonii* CCUG 27631)	1283134	1283238	*CTCATAACCCGAAGGTCGGAGGTTCAAGT CCTTCTCTCGCAACCA*AATAAGCATAAAA TCATCTTTTAAAGCACATTGTTTTAAAGCT TAAAATAATCTTACTTT	tRNA-Met (CHL_RS06785)
	1273720	1273824	CTCATAACCCGAAGGTCGGAGGTTCAAGT CCTTCTCTCGCAACCAAATAAGCATAAAA TCATCTTTTAAAGCACATTGTTTTAAAGCT TAAAATAATCTTACTTT	

[a] The start and end positions for the attachment sites refer to the nucleotide position within the contig containing the prophage genomes, except for *C. concisus* strains 13826, *C. jejuni* subsp. *doylei* 269.97 and *C. hyointestinalis* subsp. *lawsonii* CCUG 27631 which refer to the nucleotide position in the full genome

[b] Attachment sites overlapped with 3′ end of tRNA, the overlapped sequences were italic

[c] Multiple insertion sites for CON_phi prophages in *C. concisus* 13826 in which only the first attachment site (for CON_phi1) overlapped with tRNA. In NCBI database, the contig encoding JEJUNI_phiZ did not cover the full prophage genome; therefore it was unable to locate the attachment sites. No attachment site was identified in IGUA_phiZ

Conclusions

This study reports the identification of two types of *Campylobacter* Zot proteins. The high similarities of $Zot_{CampyType\_1}$ proteins suggest that they are likely to have similar virulence. $Zot_{CampyType\_2}$ proteins were less similar to each other and their virulent properties, if any, remain to be examined individually. This study provides useful information for further examination of *Campylobacter* Zot proteins as potential virulence factors.

Additional files

Additional file 1. Protein identities between prophages in other *Campylobacter* species and *C. concisus* CON_phi2 and CON_phi3. Zot proteins are in bold. Integrase proteins are underlined #Identity: Percentage of identical amino acids (number of identical amino acids divided by number of amino acids in proteins from *C. concisus* 13826).

Additional file 2. Comparison of *Campylobacter* prophage proteins with known viral proteins. #Identity: Percentage of identical amino acids (number of identical amino acids divided by number of amino acids in proteins from *Campylobacter* species).

Additional file 3. Comparison of *Campylobacter* Zot proteins with *V. cholerae* Zot and *N. meningitidis* Zot. #Identity: percentage of identical amino acids (number of identical amino acids. divided by number of amino acids of *V. cholerae* Zot or *N. meningitidis* Zot). *V. cholerae* Zot sequence Accession No. is AAF29547. *N. meningitidis* Zot sequence Accession No. is EJU63554.

Additional file 4. Comparison of Zot proteins from *Campylobacter* species, *N. meningitidis* and *V. cholerae*. * indicates identical amino acids (shaded in red). : indicates conservative mutations (shaded in blue). .indicates semi-conservative mutations. Transmembrane domains are underlined. Walker A and walker B motifs in the N-terminus of *Campylobacter* Zot proteins were identified and boxed. Walker A has a sequence of GxxxxGK[S/T], where x is any residue. Walker B motif has a sequence of hhhh[D/E], where h is a hydrophobic residue [25].

Abbreviations

IBD: inflammatory bowel disease; Zot: zonula occludens toxin; $Zot_{CampyType\_1}$: *Campylopbacter* Zot protein encoded by Cluster 1 *zot* gene; $Zot_{CampyType\_2}$: *Campylopbacter* Zot protein encoded by Cluster 2 *zot* gene; p-loop NTPase: p-loop containing nucleoside triphosphate hydrolase.

Authors' contributions

FL and HL conducted the bioinformatics analysis. LZ conceived the project. RL provided critical feedback on bioinformatics analysis. FL, LZ, HL and RL wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article (and its additional files).

Funding

This work is supported by a Faculty Research Grant awarded to Dr. Li Zhang from the University of New South Wales (Grant No: PS35329).

Received: 13 August 2016 Accepted: 8 September 2016
Published online: 15 September 2016

References

1. Lastovica AJ, On SL, Zhang L. The family Campylobacteraceae—the prokaryotes. New York: Springer; 2014. p. 307–35.
2. Lee H, Ma R, Grimm MC, Riordan SM, Lan R, Zhong L, Raftery M, Zhang L. Examination of the anaerobic growth of *Campylobacter concisus* strains. Int J Microbiol. 2014;2014:476047.
3. Zhang L, Budiman V, Day AS, Mitchell H, Lemberg DA, Riordan SM, Grimm M, Leach ST, Ismail Y. Isolation and detection of *Campylobacter concisus* from saliva of healthy individuals and patients with inflammatory bowel disease. J Clin Microbiol. 2010;48:2965–7.
4. Tanner ACR, Badger S, Lai C, Listgarten MA, Visconti RA, Socransky SS. Wolinella *gen*. nov., *WoZinella succinogenes* (*Vibrio succinogenes* Wolin et al.) comb. nov., and Description of bacteroides gracilis sp. nov., *Wolinella recta* sp. nov., *Campylobacter concisus* sp. nov., and *Eikenella corrodens* from humans with periodontal disease. Int J Syst Bacteriol. 1981;31(4):432–45.
5. Zhang L, Man SM, Day AS, Leach ST, Lemberg DA, Dutt S, Stormon M, Otley A, O'Loughlin EV, Magoffin A, et al. Detection and isolation of *Campylobacter* species other than *C. jejuni* from children with Crohn's disease. J Clin Microbiol. 2009;47:453–5.
6. Mukhopadhya I, Thomson JM, Hansen R, Berry SH, El-Omar EM, Hold GL. Detection of *Campylobacter concisus* and other *Campylobacter* species in colonic biopsies from adults with ulcerative colitis. PLoS ONE. 2011;6:e21490–1.
7. Mahendran V, Riordan SM, Grimm MC, Tran TA, Major J, Kaakoush NO, Mitchell H, Zhang L. Prevalence of *Campylobacter* species in adult Crohn's disease and the preferential colonization sites of *Campylobacter* species in the human intestine. PLoS ONE. 2011;6:e25417.
8. Lastovica AJ. Emerging *Campylobacter* spp.: the tip of the iceberg. Clin Microbiol Newsl. 2006;28:49–56.
9. Kirk KF, Nielsen HL, Thorlacius-Ussing O, Nielsen H. Optimized cultivation of *Campylobacter concisus* from gut mucosal biopsies in inflammatory bowel disease. Gut Pathog. 2016;8:27.
10. Zhang L. Oral *Campylobacter* species: initiators of a subgroup of inflammatory bowel disease? World J Gastroenterol. 2015;21:9239–44.
11. Lindblom G, Sjogren E, Hansson-Westerberg J, Kaijser B. *Campylobacter upsaliensis*, *C. sputorum sputorum* and *C. concisus* as common causes of diarrhoea in Swedish children. Scand J Infect Dis. 1995;27:187–8.
12. Nielsen H, Ejlertsen T, Engberg J, Nielsen H. High incidence of *Campylobacter concisus* in gastroenteritis in North Jutland, Denmark: a population-based study. Clin Microbiol Infect. 2013;19:445–50.
13. Kalischuk L, Inglis G. Comparative genotypic and pathogenic examination of *Campylobacter concisus* isolates from diarrheic and non-diarrheic humans. BMC Microbiol. 2011;11:53.
14. Mahendran V, Tan YS, Riordan SM, Grimm MC, Day AS, Lemberg DA, Octavia S, Lan R, Zhang L. The prevalence and polymorphisms of zonula occluden toxin gene in multiple *Campylobacter concisus* strains isolated from saliva of patients with inflammatory bowel disease and controls. PLoS ONE. 2013;8:e75525.
15. Zhang L, Lee H, Grimm MC, Riordan SM, Day AS, Lemberg DA. *Campylobacter concisus* and inflammatory bowel disease. World J Gastroenterol. 2014;20:1259–67.
16. Mahendran V, Liu F, Riordan S, Grimm M, Tanaka M, Zhang L. Examination of the effects of *Campylobacter concisus* zonula occludens toxin on intestinal epithelial cells and macrophages. Gut Pathog. 2016;8:18.
17. Bullman S, Lucid A, Corcoran D, Sleator RD, Lucey B. Genomic investigation into strain heterogeneity and pathogenic potential of the emerging gastrointestinal pathogen *Campylobacter ureolyticus*. PLoS ONE. 2013;8:1.
18. Koziel M, Lucid A, Bullman S, Corcoran GD, Lucey B, Sleator RD. Draft genome sequence of *Campylobacter corcagiensis* strain CIT045T, a representative of a novel *Campylobacter* species isolated from lion-tailed *macaques* (*Macaca silenus*). Genome Announc. 2014;2:00248–314.
19. Miller WG, Yee E, On SL, Andersen LP, Bono JL. Complete genome sequence of the *Campylobacter ureolyticus* clinical isolate RIGS 9880. Genome Announc. 2015;3(6):01291–315.
20. Miller WG, Yee E. Complete genome sequence of *Campylobacter gracilis* ATCC 33236T. Genome Announc. 2015;3(6):e01087–115.
21. Miller WG, Yee E, Huynh S, Chapman MH, Parker CT. Complete genome sequence of *Campylobacter iguaniorum* strain RM11343, isolated from an alpaca. Genome Announc. 2016;4(3):646–716.
22. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. Nucleic Acids Res. 2008;36:W5–9.
23. Mitchell A, Chang HY, Daugherty L, Fraser M, Hunter S, Lopez R, McAnulla C, McMenamin C, Nuka G, Pesseat S, et al. The InterPro protein families database: the classification resource after 15 years. Nucleic Acids Res. 2015;43:D213–21.

24. Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A, et al. The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res. 2016;44:D279–85.
25. Hanson PI, Whiteheart SW. AAA+ proteins: have engine, will work. Nat Rev Mol Cell Biol. 2005;6:519–29.
26. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Liii WZ, Lopez R, McWilliam H, Remmert M, Soding J, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol. 2011;7(1):539.
27. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–9.
28. Canchaya C, Proux C, Fournous G, Bruttin A, Brussow H. Prophage genomics. Microbiol Mol Biol Rev. 2003;67:238–76.
29. Pearson WR. An introduction to sequence similarity ("homology") searching. Current Protocols in Bioinformatics. Hoboken: John Wiley & Sons Inc; 2013. p. 311–8.
30. Kaakoush NO, Man SM, Lamb S, Raftery MJ, Wilkins MR, Kovach Z, Mitchell H. The secretome of *Campylobacter concisus*. FEBS J. 2010;277:1606–17.
31. Fasano A, Baudry B, Pumplin DW, Wasserman SS, Tall BD, Ketley JM, Kaper JB. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proc Natl Acad Sci USA. 1991;88:5242–6.
32. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011;8:785–6.
33. Bendtsen JD, Kiemer L, Fausboll A, Brunak S. Non-classical protein secretion in bacteria. BMC Microbiol. 2005;5:58.
34. Kall L, Krogh A, Sonnhammer EL. Advantages of combined transmembrane topology and signal peptide prediction-the Phobius web server. Nucleic Acids Res. 2007;35:W429–32.
35. Jackson FL, Goodman YE. *Bacteroides ureolyticus*, a new species to accommodate strains previously identified as "*Bacteroides corrodens*, anaerobic". Int J Syst Bacteriol. 1978;28:197–200.
36. Koziel M, O'Doherty P, Vandamme P, Corcoran GD, Sleator RD, Lucey B. Campylobacter corcagiensis sp. nov., isolated from faeces of captive lion-tailed macaques (*Macaca silenus*). Int J Syst Evol Microbiol. 2014;64:2878–83.
37. Miller WG, Yee E, Chapman MH. Complete genome sequences of *Campylobacter hyointestinalis* subsp. *hyointestinalis* strain LMG 9260 and *C. hyointestinalis* subsp. *lawsonii* strain LMG 15993. Genome Announc. 2016;4:00665–716.