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Clostridium perfringens: Insight into virulence evolution and population structure

Youhanna S. Sawires*, J. Glenn Songer

Department of Veterinary Science and Microbiology, University of Arizona, Room 207, 1117 East Lowell Street, Tucson AZ 85721, USA

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Abstract

Clostridium perfringens is an important pathogen in veterinary and medical fields. Diseases caused by this organism are in many cases life threatening or fatal. At the same time, it is part of the ecological community of the intestinal tract of man and animals. Virulence in this species is not fully understood and it does seem that there is erratic distribution of the toxin/enzyme genes within *C. perfringens* population. We used the recently developed multiple-locus variable-number tandem repeat analysis (MLVA) scheme to investigate the evolution of virulence and population structure of this species. Analysis of the phylogenetic signal indicates that acquisition of the major toxin genes as well as other plasmidborne toxin genes is a recent evolutionary event and their maintenance is essentially a function of the selective advantage they confer in certain niches under different conditions. In addition, it indicates the ability of virulent strains to cause disease in different host species. More interestingly, there is evidence that certain normal flora strains are virulent when they gain access to a different host species. Analysis of the population structure indicates that recombination events are the major tool that shapes the population and this panmixia is interrupted by frequent clonal expansion that mostly corresponds to disease processes. The signature of positive selection was detected in alpha toxin gene, suggesting the possibility of adaptive alleles on the other chromosomally encoded determinants. Finally, *C. perfringens* proved to have a dynamic population and availability of more genome sequences and use of comparative proteomics and animal modeling would provide more insight into the virulence of this organism.

Keywords: *Clostridium perfringens*; MLVA; Evolution of virulence; Population structure; Positive selection

1. Introduction

Clostridium perfringens is a pathogen for man and animals [1–12]. Five toxin types (A–E) are based on the existence of up to three of the four so-called major toxin (α, β, ε, ι) genes (*plc*, *cpb*, *etx*, *iap*/*ibp*) [12]. In addition, the organism produces an array of extracellular toxins and enzymes. These include beta2 toxin (*cpb2*), enterotoxin (*cpe*), perfringolysin (*pfoA*), collagenase (*colA*), lambda toxin (*lam*), hyaluronidase (*nagH*), DNase (*cadA*) [13], neuraminidases (*nanH*,*I*), and urease (*ureA-C*) (reviewed; Refs. [14,15]).

Genes (*plc*, *pfoA*, *colA*, *nagH*) are located on variable regions of the chromosome, as determined by I-*Ceu*I genome mapping [16,17], while *nanH* and *nanI* are located on a conserved region of the chromosome [18]. *cpb*, *cpb2*, *etx*, *iap*/*ibp*, *ureA-C*, and *lam* reside on plasmids of variable sizes [14,16,19,20]. In human food poisoning type A strains, *cpe* is chromosomal and resides on the 6.3 kb Tn5565 [21,22], while in non-foodborne gastrointestinal illness, it is carried on a large conjugative plasmid and linked to IS elements [23–27]. Also, *cpb*, *etx*, and *iap*/*ibp* are linked to IS*1151* [16,26,28].

Strains of toxin types B–E, are always associated with disease processes [12], indicating that they are frank pathogens. Type A strains exist as normal flora in the intestinal tract of man and animals [29–32]. However, certain strains can cause gas gangrene [33–35], food-poisoning, and gastrointestinal illness in human [26]. Moreover, necrotic enteritis in broiler chickens [36–38], enterocolitis in foals [39–43], enteritis in piglets [44–48], abomasitis and hemorrhagic enteritis in calves [49,50], and hemorrhagic enteritis in dogs [51] have been linked to type A strains.

*Corresponding author. Tel.: +1 520 626 3885; fax: +1 520 621 6366.
E-mail address: sawiresy@email.arizona.edu (Y.S. Sawires).

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Netherwood et al. [41], on surveying putative virulence determinants of type A strains isolated from foal diarrhea cases and from healthy controls, did not find evidence of a virulent sub-population causing foal diarrhea, which is distinct from isolates found in healthy animals. Wierup and DiPietro [43], found a 3–6 fold more *C. perfringens* in diarrheic than in healthy horses. Van Damme-Jongsten et al. [52], surveying 98 *C. perfringens* strains isolated from feces of different non-symptomatic animals, found 6% to possess *cpe*. Similarly, *cpb2* which is incriminated in porcine, equine, and bovine enteritis, was not detected in 10%, 90%, and 80% of type A strains isolated from these cases, respectively [53]. Moreover, alpha toxin which is suggested as key virulence attribute in necrotic enteritis in chickens [36,54], also is produced by chicken type A normal flora.

It does seem that there is erratic distribution of the toxin/enzyme genes within *C. perfringens* population(s). In addition, the outbreak nature of some type A infections (necrotic enteritis, food poisoning) as well as those caused by other toxin types [55–58], does not suggest that the organism has an opportunistic nature; rather, it has inherent capability to produce disease. Also, relying only on the major-toxin typing scheme as indicator of strain virulence does not explain the growing number of reports of type A strains as a disease causative agent.

We recently developed a multiple-locus variable-number tandem repeat analysis (MLVA) scheme for *C. perfringens* that can be used for strain typing, population genetics studies, and as a source of the phylogenetic signal [59]. In this report, we use this scheme to investigate this unique phenomenon and the evolution of virulence and population structure of this medically important pathogen.

2. Materials and methods

2.1. *C. perfringens* isolates and MLVA analysis

A total of 328 *C. perfringens* isolates were tested in this study (Table 1). Seventy-five of these were isolated from ceca of healthy food animals at slaughter ($n = 66$) or from healthy 1-day-old chicks ($n = 9$) and one colony was selected from each animal. The remainder of strains was from our collection, which consists mainly of North American clinical isolates submitted for toxin typing at the Clostridial Enteric Disease Unit (CEDU), University of Arizona. Clinical isolates included type B, C, D, and E strains from our collection. Fifteen type A clinical isolates were obtained from each host species when possible. These clinical isolates were recovered from various host species with disease state. The term "virulent" will be used throughout the manuscript to describe strains that are associated with or isolated from disease processes.

Isolates or fecal samples were plated onto brain heart infusion agar (Difco) supplemented with 5% bovine blood, 0.05% L-cysteine and incubated overnight at 37 °C in an atmosphere of 80:20 H_2:CO_2. D-cycloserine and sodium metabisulfite were added to agar plates (400 μg/mL and 1 mg/mL, respectively) to select for *C. perfringens* growth when required.

C. perfringens MLVA primers, PCR, and electrophoresis conditions were as previously described [59]. Toxin typing of normal flora strains was carried out by multiplex PCR, as described [53]. Digital images were imported into GelCompar II software v 4.01 (Applied-Maths) for image and phylogenetic analysis.

2.2. Image and phylogenetic analysis

Image and phylogenetic analysis was performed as previously described [59]. Briefly, images were normalized using the external size standard (100 bp DNA ladder, New England Biolabs) and one band was identified for each MLVA locus when present. Band-matching was initially performed with arbitrary values for optimization (1%) and position tolerance (1%), followed by calculation of optimal values for these parameters, in each of the four variable-number tandem repeats (VNTR) experiments. Band-matching data were concatenated into a single character table and each band class represented a unique allele. Dice coefficient was used to calculate the distance matrix [60] and the Neighbor-Joining (NJ) algorithm [61] to construct the phylogenetic tree. Clustering significance was tested by cophenetic correlation implemented in GelCompar software. *Clostridium difficile* JGS 370 served as an out-group to predict the root of the tree.

2.3. Minimum spanning tree (MST) for population modeling

The concatenated band-matching table was used to construct the MST, as implemented in GelCompar. Genetic distance (number of differences between two strains) was calculated using the binary coefficient. The analysis parameters included single locus variant (SLV) as priority rule [62,63], two changes as a maximum neighbor distance (double locus variant, DLV), and no missing links were allowed.

2.4. Population diversity and indices

The MLVA character table was imported into an Excel spreadsheet (Microsoft). Binary data at each MLVA locus was converted into categorical data by assigning an integer to each band class, starting at the largest molecular size and including the null allele. Population diversity, (I_A) and its correction ($\bar{r}_d$), and statistical significance (100 randomization; re-sampling without replacement) of these indices were calculated in MultiLocus v 1.3b [64,65]. These parameters investigate the structure of the population using linkage disequilibrium analysis and determine whether the number of loci used is sufficient enough to explore the population diversity.

Table 1
C. perfringens isolates

Clade[a]	Sub-clade[a]	Strain number	Toxin type	Host species	Disease	Enterotoxin	Beta2 toxin
1[b]		01E 809 MH	A	Human	Food poisoning	+	–
		01E 809 MM	A	Human	Food poisoning	+	–
		01E 810 MH	A	Human	Food poisoning	+	–
		01E 802 MA	A	Human	Food poisoning	+	–
		01E 803 YR	A	Human	Food poisoning	+	–
		JGS 1004	A	Human	Unavailable	+	–
		JGS 4151	A (strain 13)	Canine		–	+
2[b]	2-1 ($n = 97$)	JGS 1243	A	Deer	Lactic acidosis	–	–
		JGS 1714	A	Human	Unavailable	+	–
		JGS 1458	D	Ovine	Unavailable	–	–
		JGS 1323	A	Deer	Cecum	–	+
		H-5-7	A	Porcine	Normal flora	–	–
		L-13	A	Ovine	Normal flora	–	+
		JGS 4054	A	Bovine	Unavailable	–	–
		JGS 1950	A	Bovine	Abomasitis/toxemia	–	–
		JGS 1665	A	Deer	Hemorrhagic enteritis	–	–
		JGS 1540	A	Porcine	Necrotic enteritis	–	–
		JGS 1204	A	Ovine	Uterus	–	+
		JGS 1374	A	Porcine	Enteritis	–	–
		L-1	A	Ovine	Normal flora	–	+
		D-3	A	Deer	Normal flora	–	–
		JGS 4157	A	Equine	Sudden death	–	–
		D-2	A	Deer	Normal flora	–	+
		JGS 1853	A	Bovine	Sudden death	–	–
		K-2	A	Avian	Normal flora	–	–
		K-3	A	Avian	Normal flora	–	–
		K-6	A	Avian	Normal flora	–	–
		K-7	A	Avian	Normal flora	–	–
		K-9	A	Avian	Normal flora	–	–
		JGS 1892	A	Bovine	Sudden death	–	–
		JGS 1680	A	Ovine	Unavailable	–	nd
		B-4-4	A	Bovine	Normal flora	–	–
		JGS 1149	A	Caprine	Enteritis/Johne's	–	–
		JGS 1238	A	Ovine	Bronchpneumonia	–	–
		JGS 1958	A	Equine	Unavailable	–	–
		H-5-1	A	Porcine	Normal flora	–	–
		JGS 1973	A	Bovine	Sudden death	–	–
		JGS 4046	A	Feline	Enteritis	–	–
		JGS 1747	A	Ovine	Sudden death	–	nd
		JGS 1501	A	Avian	Necrotic enteritis	–	–
		JGS 4117	D	Unavailable	Unavailable	–	nd
		JGS 4059	A	Avian	Necrotic enteritis	–	+
		JGS 1985	E	Bovine	Enteritis	+	+
		JGS 4154	E	Bovine	Enteritis	+	–
		JGS 1478	E	Bovine	Enteritis	+	+
		JGS 1496	E	Bovine	Enteritis	+	+
		JGS 4071	E	Bovine	Enteritis	+	+
		JGS 1506	E	Bovine	Enteritis	+	+
		JGS 1511	E	Bovine	Enteritis	+	+
		JGS 1553	E	Bovine	Enteritis	+	nd
		JGS 1510	E	Bovine	Enteritis	+	+
		JGS 1547	E	Bovine	Enteritis	+	nd
		JGS 1792	E	Bovine	Enteritis	+	+
		JGS 1499	E	Bovine	Enteritis	+	+
		JGS 1734	A	Canine	Unavailable	–	nd
		JGS 1482	E	Bovine	Enteritis	+	+
		JGS 1739	A	Deer	Unavailable	–	–
		JGS 1187	A	Feline	Enteritis	–	+
		S-2-1	A	Ovine	Normal flora	–	+
		L-3	A	Ovine	Normal flora	–	–
		JGS 1604	A	Canine	Unavailable	–	+
		JGS 1240	D	Ovine	Bronchopneumonia	–	–

Table 1 (*continued*)

Clade[a]	Sub-clade[a]	Strain number	Toxin type	Host species	Disease	Enterotoxin	Beta2 toxin
		JGS 1612	A	Human	Enteritis	–	–
		S-1	A	Ovine	Normal flora	–	+
		JGS 1717	A	Bovine	Abomasitis	–	–
		JGS 1191	A	Equine	Unavailable	–	–
		B-4-5	A	Bovine	Normal flora	–	–
		H-5-4	A	Porcine	Normal flora	–	–
		H-5-3	A	Porcine	Normal flora	–	–
		JGS 1291	A	Equine	Unavailable	–	–
		JGS 4020	A	Canine	Unavailable	–	+
		JGS 4066	A	Avian	Necrotic enteritis	–	–
		JGS 1608	E	Bovine	Enteritis	+	+
		JGS 1901	E	Bovine	Enteritis	+	+
		JGS 1884	E	Bovine	Enteritis	+	+
		JGS 1728	E	Bovine	Enteritis	+	+
		JGS 1903	E	Bovine	Enteritis	+	+
		JGS 1521	A	Avian	Necrotic enteritis	–	+
		JGS 1888	C	Bovine	Unavailable	–	–
		JGS 1565	C	Equine	Unavailable	–	–
		JGS 1872	C	Bovine	Unavailable	–	–
		JGS 1880	C	Bovine	Unavailable	–	+
		JGS 1273	A	Equine	Unavailable	–	+
		JGS 1301	A	Equine	Unavailable	–	–
		H-4-2	A	Porcine	Normal flora	–	–
		JGS 1825	A	Bovine	Enteritis	–	–
		JGS 1558	D	Caprine	Enteritis	–	–
		JGS 1900	A	Canine	Unavailable	–	–
		JGS 1250	A	Porcine	Bronchopneumonia	–	–
		I-3	A	Emu	Normal flora	–	–
		JGS 1771	A	Bovine	Enteritis	–	nd
		JGS 1719	A	Human	Unavailable	+	–
		JGS 4150	C	Porcine	Unavailable	–	+
		JGS 1711	A	Human	Unavailable	+	+
		JGS 1325	A	Equine	Jejunum	–	+
		JGS 1712	A	Human	Unavailable	+	–
		JGS 1047	A	Ovine	Enterotoxemia	–	–
		JGS 1537	A	Porcine	Enteritis/diarrhea	–	–
		H-3-1	A	Porcine	Normal flora	–	+
		JGS 1681	A	Alpaca	Unavailable	–	–
		JGS 1706	C	Unavailable	Unavailable	–	nd
		JGS 1727	C	Unavailable	Unavailable	–	–
		JGS 1523	C	Porcine	Unavailable	–	–
		JGS 1926	C	Seed culture	Unavailable	–	–
	2-2-1($n = 66$)	JGS 1277	A	Ovine	Unavailable	–	–
		JGS 1307	A	Porcine	Malabsorbtion	–	–
		JGS 1942	D	Caprine	Sudden death	–	–
		JGS 1941	D	Caprine	Unavailable	–	–
		JGS 4091	A	Equine	Enteritis	–	–
		JGS 1148	A	Porcine	Enteritis/salmonellosis	–	+
		B-2-1	A	Bovine	Normal flora	–	+
		JGS 1182	D	Ovine	Sudden death	–	–
		JGS 1198	A	Feline	bile duct	–	+
		L-9	A	Ovine	Normal flora	–	+
		JGS 1635	A	Feline	Sudden death	–	nd
		L-5-3	A	Ovine	Normal flora	–	–
		L-5-4	A	Ovine	Normal flora	–	–
		JGS 1332	A	Deer	Small intestine	–	+
		JGS 1013	A	Deer	Unavailable	–	–
		H-5-10	A	Porcine	Normal flora	–	–
		JGS 1384	A	Equine	Sheath	–	+
		JGS 1370	A	Equine	Umbilicus	–	–
		JGS 1986	E	Bovine	Enteritis	+	+
		JGS 4158	D	Caprine	Enteritis/septicemia	+	+
		L-2	A	Ovine	Normal flora	–	+

Table 1 (*continued*)

Clade[a]	Sub-clade[a]	Strain number	Toxin type	Host species	Disease	Enterotoxin	Beta2 toxin
		JGS 4080	A	Bovine	Septicemia	–	–
		JGS 4058	A	Llama	Unavailable	–	–
		JGS 1869	A	Bovine	Enteritis	–	–
		JGS 1090	C	Porcine	Unavailable	–	+
		JGS 1672	C	Porcine	Unavailable	–	+
		JGS 1691	C	Porcine	Unavailable	–	+
		JGS 1905	C	Porcine	Enteritis	–	+
		S-3	A	Ovine	Normal flora	–	+
		S-4	A	Ovine	Normal flora	–	+
		JGS 1736	A	Bovine	Enteritis	–	–
		JGS 1614	A	Equine	Unavailable	–	–
		JGS 1703	A	Bovine	Enteritis	–	–
		H-3	A	Porcine	Normal flora	–	+
		JGS 1705	D	Ovine	Unavailable	–	–
		JGS 1663	A	Ovine	Unavailable	–	–
		B-4-1	A	Bovine	Normal flora	–	–
		JGS 1509	D	Caprine	Unavailable	–	nd
		JGS 1146	A	Porcine	Bronchopneumonia	–	–
		JGS 1810	A	Alpaca	Sudden death	–	nd
		JGS 1602	A	Alpaca	Sudden death	–	–
		JGS 1840	A	Alpaca	Sudden death	–	nd
		JGS 1819	A	Alpaca	Sudden death	–	–
		JGS 1764	A	Llama	Unavailable	–	nd
		JGS 1613	A	Alpaca	Sudden death	–	–
		JGS 1768	D	Unavailable	Unavailable	–	–
		JGS 1841	D	Unavailable	Unavailable	–	nd
		JGS 1579	A	Feline	Sudden death	–	–
		JGS 1304	A	Ovine	Enterotoxemia	–	–
		JGS 4013	A	Llama	Unavailable	–	–
		D-8	A	Deer	Normal flora	–	+
		JGS 4135	A	Avian	Necrotic enteritis	–	+
		H-5-2	A	Porcine	Normal flora	–	–
		H-4-4	A	Porcine	Normal flora	–	+
		H-4-5	A	Porcine	Normal flora	–	+
		JGS 4143	A	Avian	Necrotic enteritis	–	+
		JGS 4095	A	Equine	Enterocolitis	+	–
		D-7	A	Deer	Normal flora	–	+
		H-5-8	A	Porcine	Normal flora	–	–
		L-11	A	Ovine	Normal flora	–	+
		H-4-1	A	Porcine	Normal flora	–	+
		JGS 1826	A	Bovine	Enteritis	–	–
		JGS 1217	A	Feline	Leukemia	–	–
		JGS 4141	A	Avian	Necrotic enteritis	–	+
		JGS 1491	A	Equine	Necrotic enteritis	+	+
		L-4	A	Ovine	Normal flora	–	–
	2-2-2-1($n = 96$)	D-1	A	Deer	Normal flora	–	+
		H-1	A	Porcine	Normal flora	–	–
		K-5	A	Avian	Normal flora	–	–
		I-4	A	Emu	Normal flora	–	–
		H-2-2	A	Porcine	Normal flora	–	–
		JGS 1657	A	Avian	Unavailable	–	–
		JGS 4099	A	Equine	Enteritis	–	–
		K-4	A	Avian	Normal flora	–	–
		L-7	A	Ovine	Normal flora	–	+
		L-8	A	Ovine	Normal flora	–	+
		JGS 1320	A	Canine	Reproductive tract	–	+
		JGS 1313	A	Canine	Small intestine	–	+
		JGS 1338	A	Canine	Conjunctiva	–	+
		JGS 4152	D	Ovine	Pulpy kidney	+	+
		JGS 1218	A	Ovine	Rib fractures	–	+
		JGS 1842	A	Equine	Hemorrhagic colitis	–	+
		H-4-3	A	Porcine	Normal flora	–	+
		JGS 1696	C	Unavailable	Unavailable	–	+

Table 1 (*continued*)

Clade[a]	Sub-clade[a]	Strain number	Toxin type	Host species	Disease	Enterotoxin	Beta2 toxin
		JGS 1460	C	Porcine	Unavailable	–	+
		JGS 1414	A	Canine	Enteritis	+	–
		JGS 1527	C	Porcine	Unavailable	–	+
		JGS 1376	A	Canine	Conjunctiva	–	+
		JGS 1244	A	Ovine	Enteritis/salmonellosis	–	–
		JGS 1938	A	Bovine	Sudden death	–	–
		JGS 1357	A	Canine	Chronic colitis	–	–
		JGS 1693	A	Bovine	Enteritis	–	–
		JGS 1946	D	Caprine	Unavailable	–	–
		JGS 1837	A	Alpaca	Sudden death	–	–
		JGS 1836	A	Alpaca	Sudden death	–	–
		JGS 1910	C	Bovine	Enteritis	–	+
		JGS 1533	A	Bovine	Unavailable	–	–
		JGS 1943	E	Bovine	Enteritis	+	+
		JGS 4145	A	Equine	Unavailable	+	+
		JGS 1076	C	Porcine	Enteritis	–	+
		JGS 1070	C	Porcine	Enteritis	–	+
		JGS 1071	C	Porcine	Enteritis	–	+
		JGS 1075	C	Porcine	Enteritis	–	+
		JGS 4006	A	Cervid	Unavailable	–	–
		JGS 1676	A	Avian	Unavailable	–	–
		JGS 1551	D	Ovine	Unavailable	–	–
		JGS 1948	D	Caprine	Enterotoxemia	–	–
		JGS 4139	D	Caprine	Sudden death	+	+
		JGS 4138	D	Caprine	Sudden death	+	+
		JGS 1902	D	Ovine	Enteritis	+	+
		JGS 1791	A	Deer	Unavailable	–	nd
		JGS 4111	C	Bovine	Sudden death	–	+
		JGS 1637	A	Equine	Hemorrhagic enteritis	–	–
		JGS 1015	C	Bovine	Unavailable	–	–
		JGS 1022	C	Canine	Food	+	nd
		JGS 1556	A	Alpaca	Unavailable	–	–
		JGS 1928	A	Canine	Unavailable	–	–
		JGS 1544	C	Porcine	Unavailable	–	+
		JGS 1813	C	Unavailable	Unavailable	–	+
		JGS 1975	E	Bovine	Enteritis	+	+
		JGS 1413	A	Canine	Enteritis	+	–
		JGS 1874	C	Porcine	Unavailable	–	–
		JGS 1142	C	Bovine	Acute enteritis	–	–
		JGS 1284	A	Equine	Umbilicus	–	+
		JGS 1721	D	Ovine	Enteritis	–	–
		JGS 4061	A	Caprine	Necrotic enteritis	–	–
		B-3-1	A	Bovine	Normal flora	–	+
		H-2-1	A	Porcine	Normal flora	–	–
		K-8	A	Avian	Normal flora	–	–
		L-5	A	Ovine	Normal flora	–	+
		JGS 1206	A	Equine	Lung	–	–
		JGS 4043	A	Avian	Necrotic enteritis	–	–
		D-5	A	Deer	Normal flora	–	+
		B-3-4	A	Bovine	Normal flora	–	–
		L-6	A	Ovine	Normal flora	–	+
		JGS 1118	B	Ovine	Unavailable	–	–
		JGS 4105	D	Ovine	Sudden death	–	nd
		JGS 1620	A	Avian	Unavailable	–	–
		JGS 1677	A	Avian	Unavailable	–	–
		JGS 1984	B	Unavailable	Unavailable	–	–
		JGS 1769	A	Canine	Unavailable	+	nd
		JGS 1271	A	Equine	Abscess	–	+
		JGS 1644	A	Feline	Normal	–	–
		JGS 1181	A	Ovine	Intestine	–	nd
		H-2-3	A	Porcine	Normal flora	–	–
		JGS 4106	C	Porcine	Unavailable	–	–
		JGS 1164	C	Bovine	Enteritis	–	–
		JGS 1622	A	Cervid	Unavailable	–	nd

Table 1 (*continued*)

Clade[a]	Sub-clade[a]	Strain number	Toxin type	Host species	Disease	Enterotoxin	Beta2 toxin
		JGS 1640	C	Porcine	Unavailable	–	+
		JGS 1713	A	Human	Unavailable	+	+
		JGS 4175	A(ATCC13124)	Human			–
		JGS 1343	A	Equine	Hoof	–	+
		JGS 1805	A	Cervid	Unavailable	–	–
		JGS 1228	A	Caprine	Enteritis	–	–
		S-5	A	Ovine	Normal flora	–	+
		JGS 4003	A	Bovine	Sudden death	–	+
		JGS 1832	A	Alpaca	Sudden death	–	nd
		JGS 1927	D	Unavailable	Unavailable	–	–
		JGS 1949	D	Caprine	Unavailable	–	–
		JGS 1944	D	Caprine	Enterotoxemia	–	–
		JGS 1328	A	Ovine	Intestine	–	–
		JGS 1945	D	Caprine	Enteritis	–	–
	2-2-2-2($n = 62$)	L-14	A	Ovine	Normal flora	–	+
		JGS 1232	A	Equine	Hemorrhagic enteritis	–	–
		JGS 1363	A	Canine	Enteritis	–	+
		H-5-6	A	Porcine	Normal flora	–	–
		H-4	A	Porcine	Normal flora	–	–
		JGS 4036	A	Bovine	Enteritis	–	–
		JGS 1796	A	Bovine	Enteritis	–	nd
		JGS 1183	A	Ovine	Sudden death	–	–
		JGS 4119	A	Caribou	Enteritis	–	+
		B-6	A	Bovine	Normal flora	–	–
		JGS 1987	E	Bovine	Enteritis	+	+
		D-4	A	Deer	Normal flora	–	+
		JGS 1415	A	Canine	Enteritis	+	+
		JGS 1846	A	Alpaca	Sudden death	–	–
		H-4-6	A	Porcine	Normal flora	–	–
		JGS 1642	A	Porcine	Enteritis/diarrhea	–	–
		JGS 1394	A	Feline	Unavailable	–	–
		JGS 1809	A	Feline	Enteritis	–	–
		JGS 1239	A	ovine	Peritonitis	–	–
		JGS 4042	A	Avian	Necrotic enteritis	–	–
		B-4-3	A	Bovine	Normal flora	–	–
		L-5-1	A	Ovine	Normal flora	–	–
		L-12	A	Ovine	Normal flora	–	–
		H-5-5	A	Porcine	Normal flora	–	–
		H-5-9	A	Porcine	Normal flora	–	–
		B-3-2	A	Bovine	Normal flora	–	–
		L-10	A	Ovine	Normal flora	–	–
		NCTC 8239	A	Human	Food poisoning	+	–
		JGS 1292	A	Human	Unavailable	+	–
		FD 1041	A	Human	Food poisoning	+	–
		JGS 1549	A	Elk	Unavailable	–	nd
		JGS 1546	A	Canine	Unavailable	–	nd
		JGS 1147	A	Porcine	Enteritis/swine dysentery	–	+
		JGS 1652	A	Porcine	Necrotic enteritis	–	+
		JGS 1165	A	Porcine	Ascites	–	nd
		JGS 1531	A	Porcine	Enteritis/diarrhea	–	+
		JGS 1715	A	Porcine	Enteritis/diarrhea	–	+
		JGS 4172	C	Porcine	Unavailable	–	+
		JGS 1169	A	Porcine	Ascites	–	+
		JGS 1543	C	Porcine	Unavailable	–	+
		JGS 1495	C	Porcine	Unavailable	–	+
		JGS 1504	C	Porcine	Necrotic enteritis	–	+
		L-5-2	A	Ovine	Normal flora	–	–
		JGS 1475	C	Porcine	Unavailable	–	+
		JGS 1988	C	Porcine	Unavailable	–	+
		JGS 1562	C	Unavailable	Unavailable	–	+
		JGS 1564	C	Porcine	Unavailable	–	+
		JGS 1659	C	Porcine	Enteritis	–	+
		JGS 1508	C	Porcine	Unavailable	–	+

Table 1 (*continued*)

Clade[a]	Sub-clade[a]	Strain number	Toxin type	Host species	Disease	Enterotoxin	Beta2 toxin
		JGS 1802	C	Unavailable	Unavailable	−	+
		JGS 1756	C	Porcine	Unavailable	−	+
		JGS 1729	C	Porcine	Unavailable	−	+
		JGS 1800	C	Unavailable	Unavailable	−	+
		JGS 1897	C	Porcine	Unavailable	−	+
		JGS 1816	A	Ovine	Hemorrhagic enteritis	−	+
		JGS 1205	A	Feline	Conjunctiva	−	−
		JGS 4032	A	Canine	Unavailable	+	nd
		K-1	A	Avian	Normal flora	−	−
		JGS 1742	A	bovine	Enteritis	−	−
		JGS 1587	A	Porcine	Hemorrhagic enteritis	−	−
		B-5	A	Bovine	Normal flora	−	−
		H-2	A	Porcine	Normal flora	−	−

n, number of strains in each sub-clade; nd, not done; +, present; −, absent.
[a]Strains are displayed according to the order seen on the NJ tree.
[b]Clade is a biological group of strains with a common evolutionary ancestor.

2.5. Detection of positive selection

Selective neutrality of the putative virulence genes was tested using the McDonald–Kreitman (MK) test as, implemented in DnaSP v 4.10 [66,67]. Neutrality index (NI) is calculated from the MK 2×2 table, and it indicates the direction and degree of departure from selective neutrality [68]. Only genes with more than one entry and show sequence polymorphism/divergence and a homologue in another clostridial species in the NCBI database, were included in this analysis [69]. The sequence similarity search was performed by BLASTP/N and sequence alignment was carried out by Clustal W [70], as implemented in BioEdit program [71]. Nucleotide sequence accession numbers were for *plc* (D10248, L43545, M24904, X17300, X13608, D32123, D32124, D32126, D32128, D49968, D49969, L43547, L43548, D63911, BA000016, AY823400, AF204209) and *colA* (D13791, BA000016). *Clostridium novyi plc* (D32125) and *Clostridium histolyticum colG* (D87215, AB026889) served as out-group for *C. perfringens plc* and *colA*, respectively.

3. Results

3.1. Isolation and toxin typing of normal flora strains

The animals were healthy at slaughter, and there was no evidence of inflammation in their ceca. All normal flora isolates are type A strains and nearly 45% carry *cpb2* ($n = 29$). Sixty-five percent (15/23) of the ovine normal flora strains have *cpb*2, while deer normal flora have the highest proportion at 85% (6/7). Twenty-five percent and 20% of porcine (6/24) and bovine (2/10) normal flora strains, respectively, carry *cpb*2. None of the avian normal flora strains contain *cpb*2.

3.2. C. perfringens NJ phylogenetic tree

Correlation between tree-driven similarity and matrix similarity is 0.63 at the root of the tree and reach 1 at external nodes. *C. perfringens* strains are phylogenetically clustered over two major clades (Fig. 1, Table 1). Branches that connect internal nodes are short, indicating small genetic distance. Toxin types A, C, and D strains are dispersed on the tree without a specific pattern, while type E strains ($n = 18$) exist on one major sub-clade, 2-1, with exception of JGS1986, 1943, 1975, and 1987, which are located on different sub-clades. All clones (groups of at least two strains which match at the five MLVA loci, indicating a single recent ancestor) are made up of strains belonging to a single toxin type, except one at sub-clade 2-2-2-1 (Fig. 1, Table 1), which contains a bovine type C strain and a deer type A clinical strain. Also, except for type E (which is always recovered from bovine host with enteritis; $\chi^2 = 8.9$, $P<0.01$), there is no evidence of association between strains' phylogeny and the host, disease, or toxin type.

Clade 1 contains mainly human food poisoning strains submitted for MLVA analysis during the course of this study. They have a common ancestor, but a single recent ancestor was detected in case of 01E-809MM and 01E-810MH, and in case of 01E-802MA and 01E-803YR. In addition, 01E-802MA and 01E 803YR show phylogenetic relatedness to *C. perfringens* strain 13 (JGS4151).

The two type B strains exist on the same phylogenetic cluster (sub-clade 2-2-2-1) with JGS1118, sharing a common ancestor with a type D ovine enteritis strain, while JGS1984 share a common ancestor with a type A strain from a canine clinical case.

Type A clinical isolates from different host species and diseases either share common ancestor(s) or phylogenetic relationship(s) among themselves or with strains of other toxin types. Among 9 strains isolated from poultry with necrotic enteritis, three (JGS4059, 4066, 1521) are phylogenetically related to type E strains isolated from bovine enteritis cases. JGS4135 (Bird 89 strain) clustered phylogenetically with a porcine normal flora strain (H-5-2), JGS4141 with an equine type A necrotic enteritis

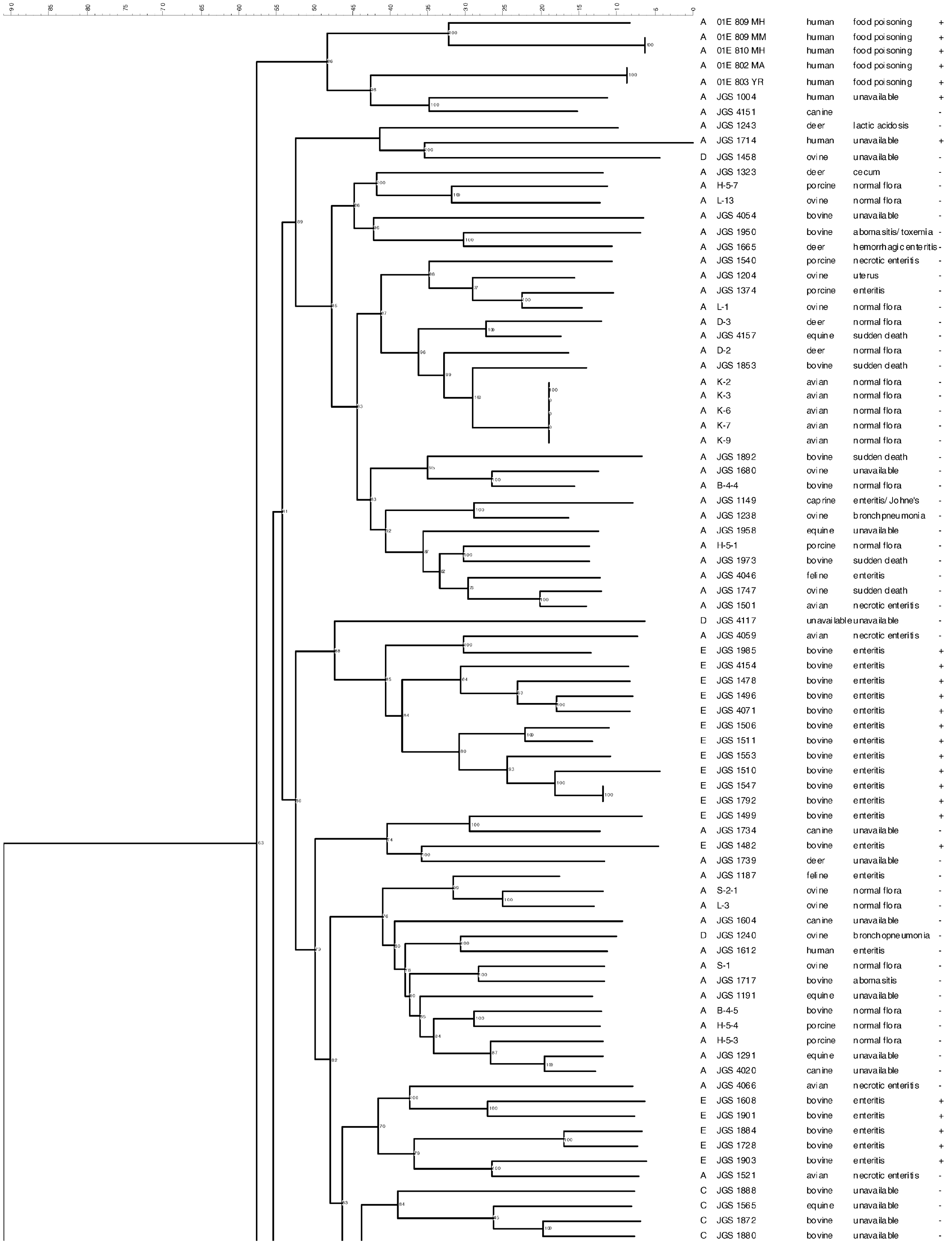

Fig. 1. *C. perfringens* phylogenetic tree. The scale on the top of the figure is genetic distance and the tree is additive meaning the branches are not equidistant from the root. The distance between two operational taxonomic units, OTUs (strains) equal the sum of lengths of all the branches connecting them. Toxin-type, strain number, host species, disease, and presence or absence of enterotoxin and beta2 toxin is displayed on the tree.

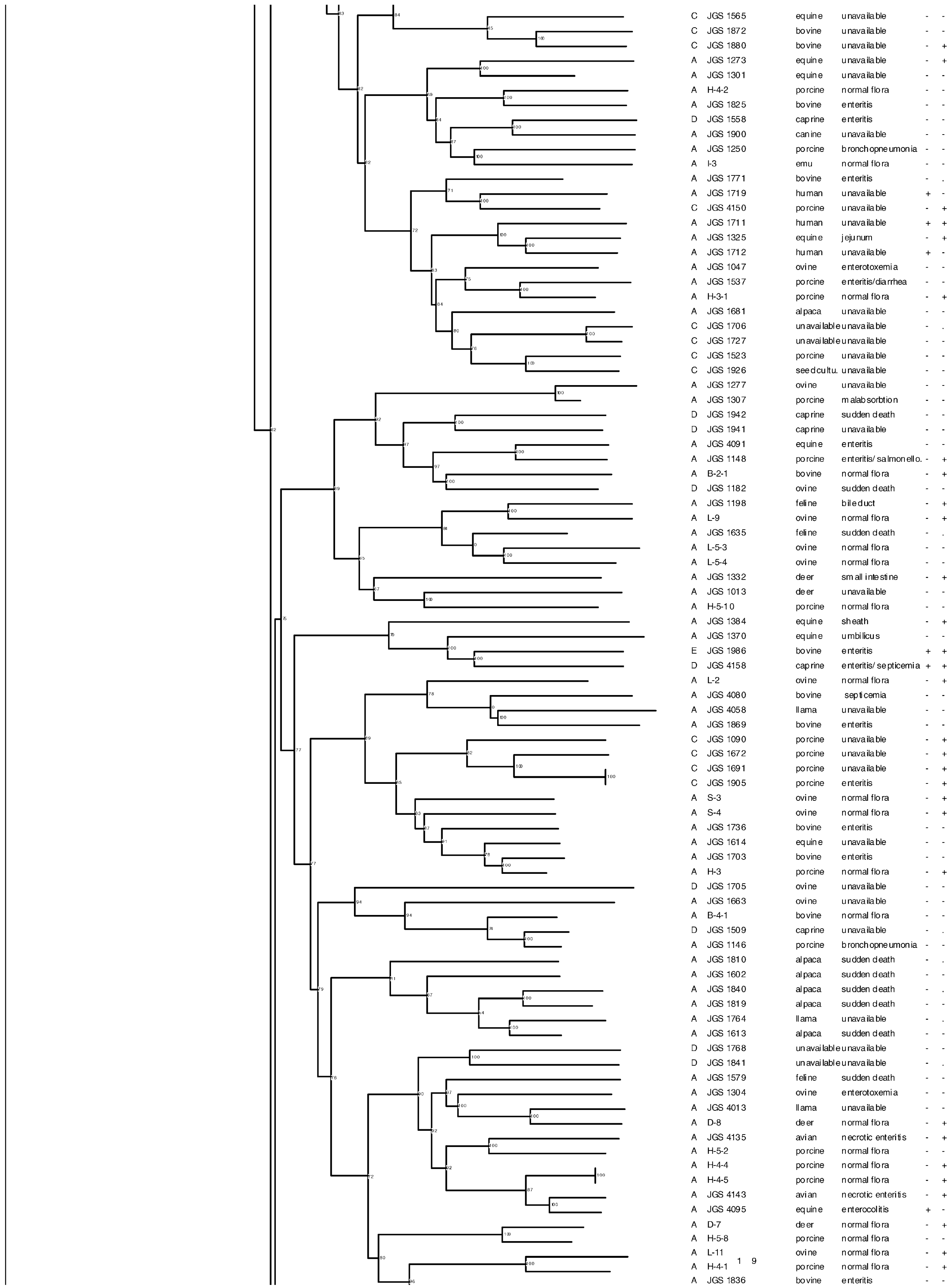

Fig. 1. (*Continued*)

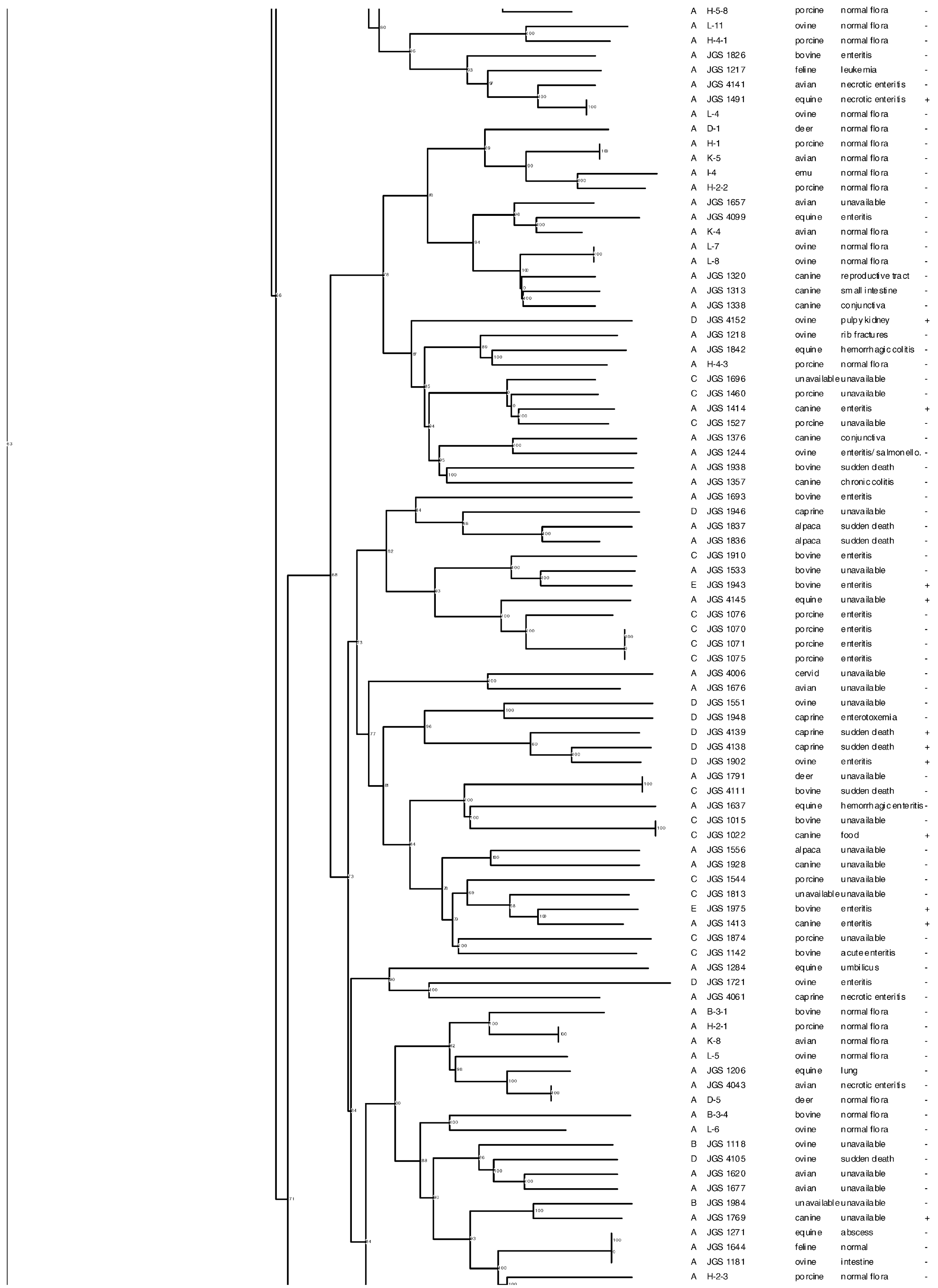

Fig. 1. (*Continued*)

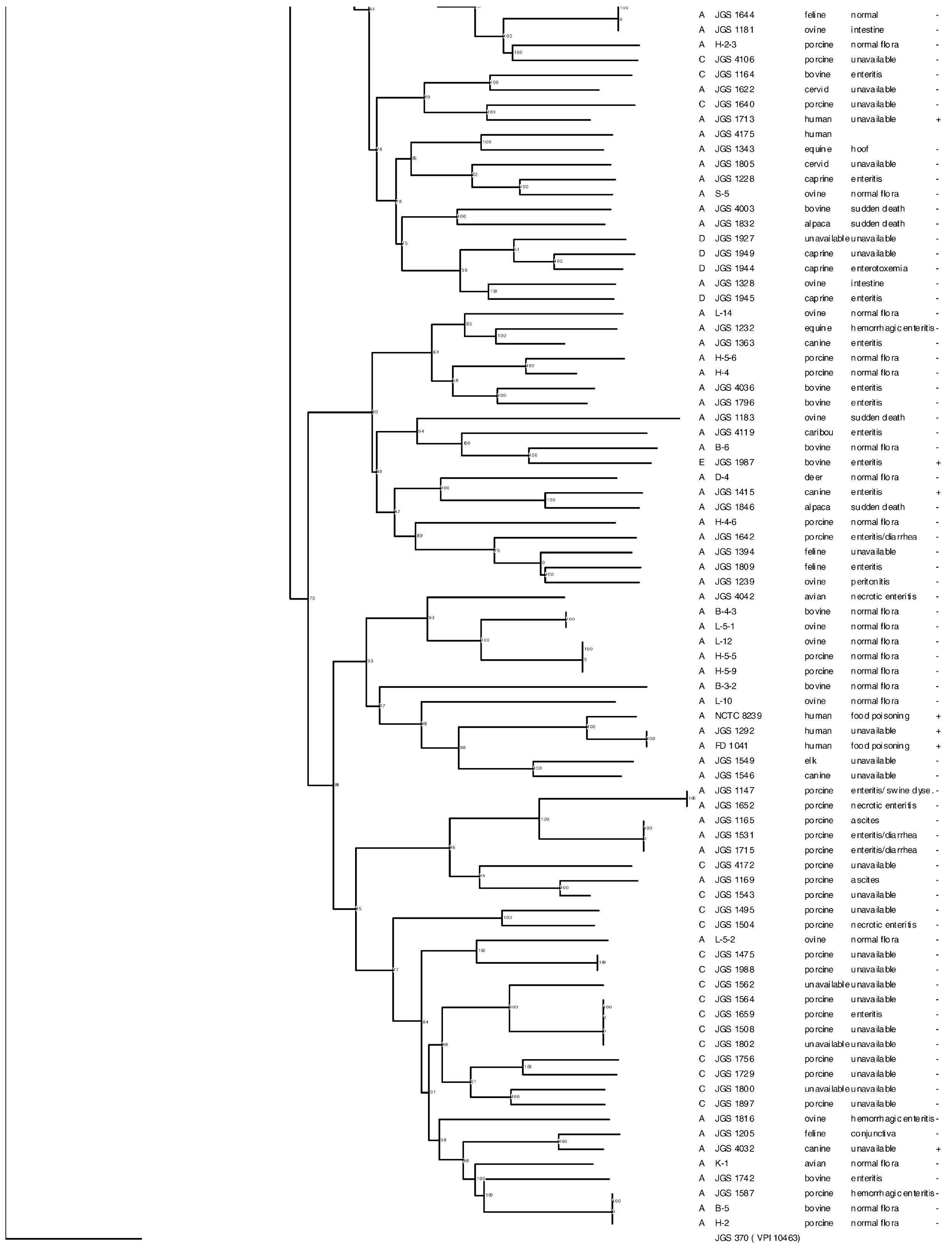

Fig. 1. (*Continued*)

strain (JGS1491), JGS4143 with an equine type A enterocolitis strain (JGS4095), JGS1501 with an ovine type A sudden death strain (JGS1747), and JGS4042 with a bovine normal flora strain (B-4-3). JGS 4043 shares a single recent ancestor with a deer normal flora strain.

Interestingly, normal flora strains showed no evidence of association between strains' phylogeny and host (chicken flora $\chi^2 = 0.11$, $0.95 < P < 0.8$); rather, normal flora from different mammalian or avian hosts exist in the same cluster or even clone. Moreover, certain flora strains from specific host species have close phylogenetic relationships with or share a single recent ancestor with virulent strains of different host species or different toxin types. Of the 24 porcine flora strains examined, 6 are phylogenetically close to virulent strains isolated from non-porcine hosts, two have the same relationship with porcine virulent strains and one shares a single recent ancestor with a type C strain of porcine origin. Similarly, two bovine flora strains share close phylogeny with two type D strains from ovine and caprine hosts, two have close phylogeny with virulent type A strains of non-bovine origin, and one shares phylogeny with a type E strain. The theme is the same for poultry flora ($n = 1$), emu flora ($n = 1$), deer flora ($n = 4$), and ovine flora ($n = 9$), which share phylogeny either with virulent or different toxin type strains of non-poultry, non-emu, non-deer, and non-ovine origin, respectively.

3.3. *C. perfringens* minimum spanning tree

Unlike phylogenetic trees, MST searches for the founding genotype or ancestor. This algorithm assumes that recombination in bacterial populations is sufficient to break clonal evolution to the degree that history cannot be displayed efficiently as a tree, but rather as a network relationship; this recombination is interrupted by bouts of clonal expansion [62,63,72,73]. The algorithm identified 22 clonal complexes (isolates identical, SLV, or DLV, from one another), most of them composed of a maximum of 5 strains either from the same or different toxin types (Fig. 2).

Clonal complex 1 comprises 149 isolates with K-4, an avian flora strain, as the founding genotype (Fig. 3). Virulent type A strains from different host species, normal flora strains, and strains of toxin types other than A, were detected in this clonal complex. For instance, JGS4099 genotype, an equine enteritis isolate, is the ancestor of four type A virulent strains, three type C strains, and two normal flora strains. Similarly, H-3, a porcine normal flora strain, is the founding genotype of a group containing 5 virulent type A strains and five normal flora strains (Fig. 4).

The human food poisoning strains in clade-1 (clonal complex 8; data not shown) revealed 01E-809MH as the founding genotype for this group. Likewise, JGS4095, an equine enterocolitis strain, is the founding genotype of a group containing H-4-4, JGS1491, and three of the poultry necrotic enteritis strains (JGS4143, 4141, 4135). Poultry necrotic enteritis strain JGS1501 is the founding genotype of JGS1747, B-4-3 is the founding genotype of a poultry necrotic enteritis strain (JGS4042), while JGS4043 formed a clone with D-5, a deer normal flora strain. The remaining poultry necrotic enteritis strains showed relationships similar to those displayed on the NJ tree, but were not included in a complex with type E strains because of the stringent parameters used to construct the MST.

Interestingly, acquisition/loss of major toxin genes is evident in this complex (Fig. 5). For example, the MLVA genotype of JGS1769, a canine type A clinical isolate is identified as the ancestor of JGS1984, a type B strain. The genotype of JGS 1413, a canine type A enteritis isolate, is the ancestor of JGS1975 (type E) and JGS1813 (type C), respectively. Similarly, JGS1363 genotype, a canine type A clinical isolate, is the ancestor of JGS1543, a porcine type C strain, which in turn is the ancestor of JGS1169, a porcine type A strain isolated from ascites case. Also, the genotype of K-1, an avian flora strain is the ancestor of JGS1182, a type D strain. Similar evidence of acquisition/loss of *cpe* and *cpb2* is also evident in clonal complex 1 (data not shown).

This acquisition/loss of these toxin genes is, in many cases, associated with switching to a different host or even to a different micro-environment or niche. For example, acquisition/loss of *cpb*, as the organism switched to extraintestinal environment, demonstrated in the above-mentioned situation. Similarly, a *cpe* negative H-1 genotype, a porcine normal flora, is the ancestor of *cpe* positive JGS4095 genotype isolated from equine enterocolitis, which in turn is the ancestor of a *cpe* negative porcine normal flora strain, H-4-4. Likewise, K-2, an avian *cpb2* negative strain, is the founding genotype of H-3, a porcine *cpb2* positive strain, which in turn is the ancestor of JGS1614, an equine *cpb2* negative clinical isolate. However, some cases include loss of these genes within the same host species; for example, JGS1943, a type E strain, is the founding genotype of JGS1533, a type A strain isolated from a bovine clinical case (clonal complex 16; data not shown). Likewise, H-3-1, a *cpb2* positive porcine flora strain is the founding genotype of JGS1537, a *cpb2* negative porcine enteritis strain.

3.4. *C. perfringens* population structure

Analysis of the genotypic diversity versus the number of loci indicated that scoring more MLVA loci would not increase the genotypic diversity; it reached a plateau (Fig. 6). Linkage disequilibrium analysis of the population was performed on different levels as previously suggested [65]. All isolates, all linkage groups showed that *C. perfringens* population is panmictic ($P < 0.01$; Table 2). The same results were achieved at the level of sub-clades where departure from equilibrium is rejected. However, sub-clade 2-1 showed evidence of clonality ($P = 0.01$), indicating that an epidemic population may have caused this temporary disequilibrium. Repeating the analysis without the 12 type E strains in one of the sub-clade 2-1 clusters resulted in the disappearance of this disequilibrium ($I_A = 0.0538$; $\bar{r}_d = 0.0144$; $P = 0.21$). Departure from panmixia was evident when type C and E were analysed separately but not in type D strains (Table 2).

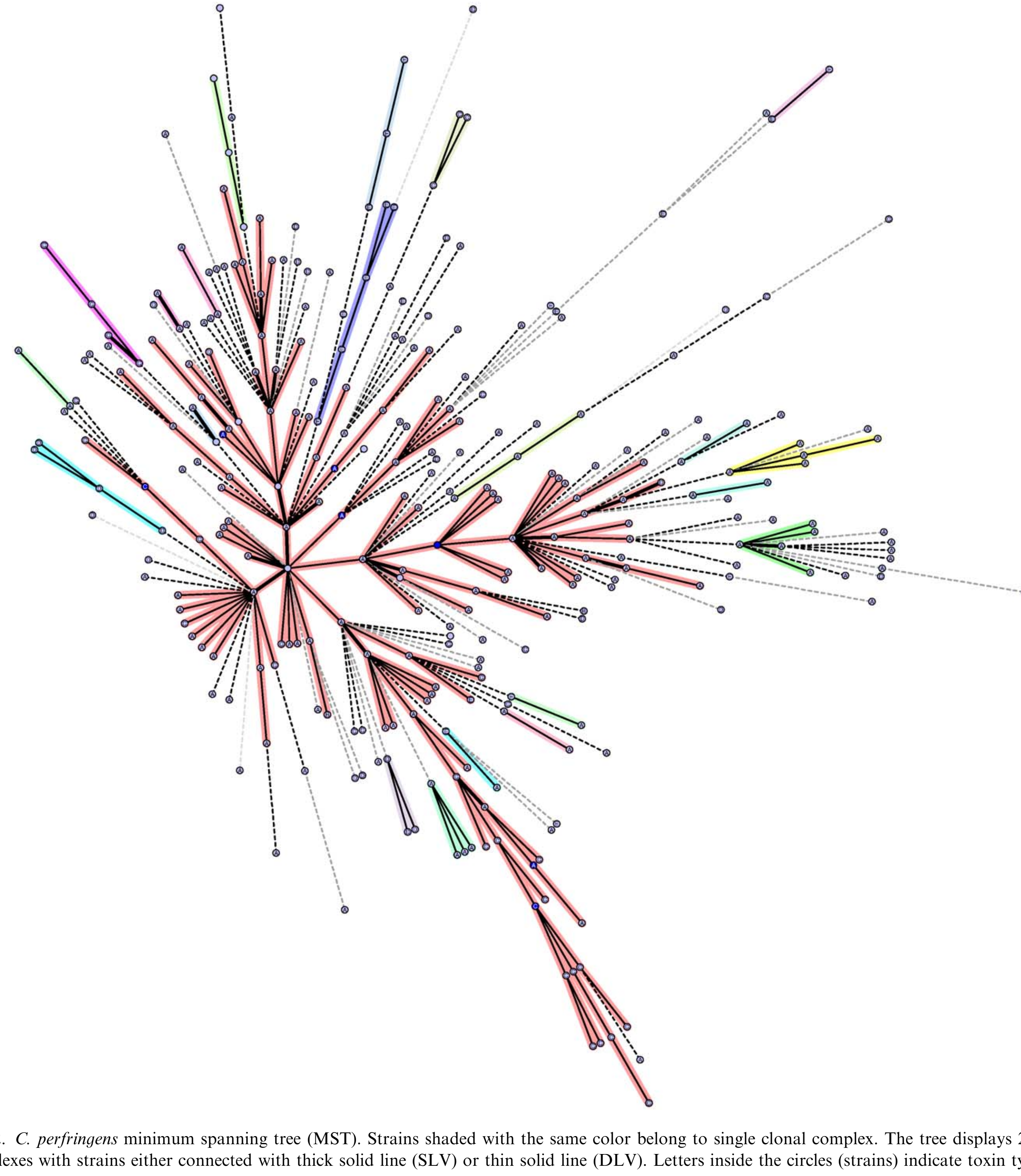

Fig. 2. *C. perfringens* minimum spanning tree (MST). Strains shaded with the same color belong to single clonal complex. The tree displays 22 clonal complexes with strains either connected with thick solid line (SLV) or thin solid line (DLV). Letters inside the circles (strains) indicate toxin type.

3.5. Detection of selection

The McDonald and Kreitman test contrasts the ratio of non-synonymous replacements to synonymous substitutions within-species and between species and use this to reject the null hypothesis of selective neutrality [66,74]. A significant difference between these two ratios indicates the existence of selection, while the number of fixed non-synonymous/synonymous substitutions determines whether it is a purifying or positive selection. Similarly, NI, a qualitative indicator, will have a value of 1 under strict neutrality. *plc* showed evidence of adaptive evolution ($P = 0.047$) with NI deviated from value of 1; that is expected under neutrality. In the case of *colA*, the neutral mutation hypothesis could not be rejected, indicating that alleles at this locus are selectively neutral or nearly neutral (Table 3). However, this test assumes that there is no recombination at each locus, as this might induce sequence variability resembling molecular adaptation [75]. Thus, these results should be viewed with some caution.

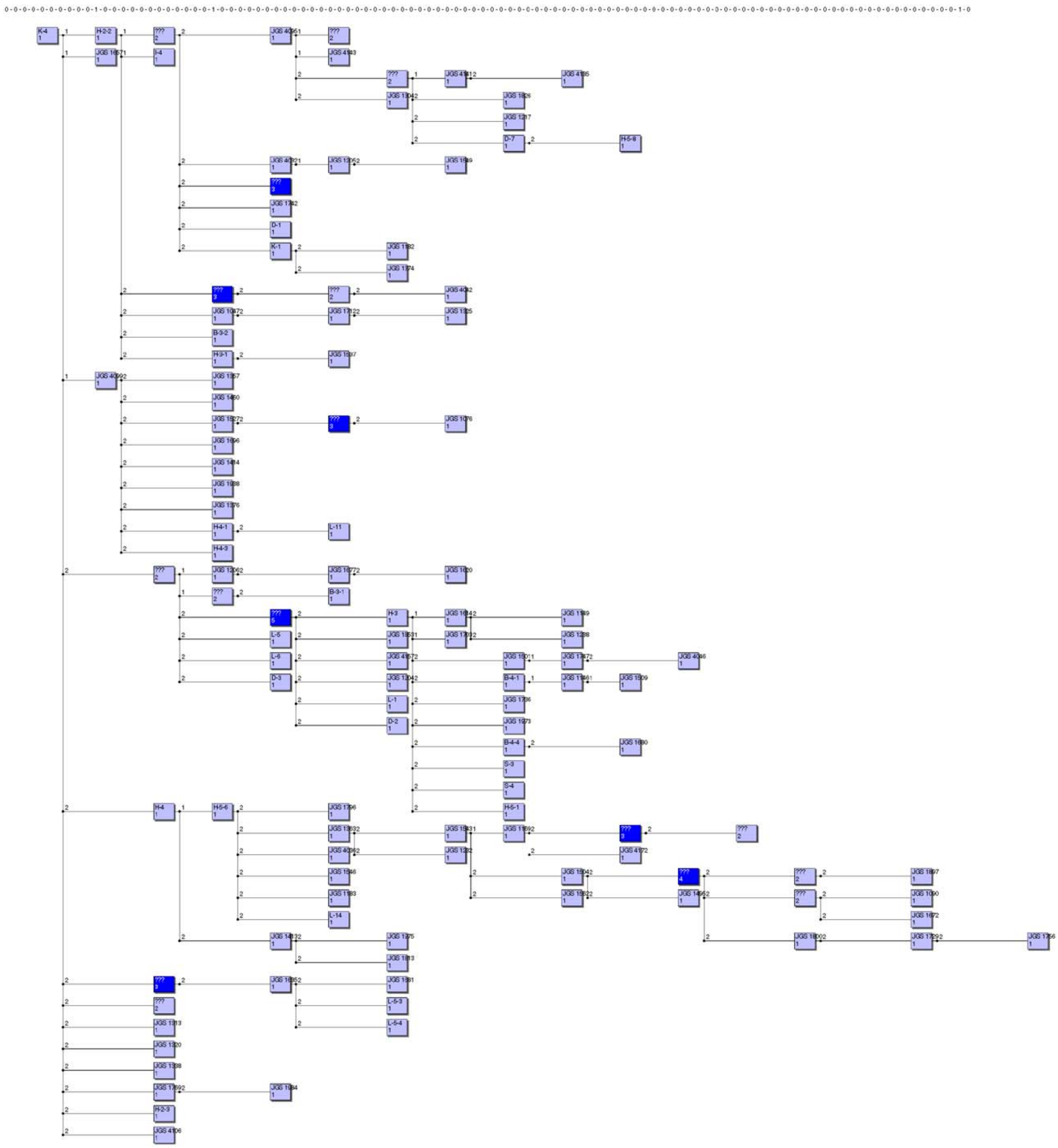

Fig. 3. Clonal complex 1; strain number. The complex displays strain numbers and shows founding genotypes. Relations between strains are uni-directional starting from the founding genotype. Each rectangle shows strain(s) number(s) and shaded blue if more than 2 strains form a clone. Numbers above the lines indicate distance.

4. Discussion

C. perfringens is an important pathogen in veterinary and medical fields. Diseases caused by this organism are in many cases life threatening or fatal [1,12]. In addition, it is part of the ecological community of the intestinal tract of man and other animals [29–31].

The virulence in this species is not fully understood. However, virulence of type C strains is attributed to beta toxin, type D strains to epsilon toxin, type E to iota toxin, and type B to beta and epsilon toxins [76–79]. Likewise, virulence of type A gas gangrene strains is credited to alpha toxin [1], and virulence of type A food poisoning or gastrointestinal illness strains is attributed to *C. perfringens* enterotoxin [80].

The role of these genes in producing the pathological picture of *C. perfringens* diseases and syndromes is evident by their phenotype in vitro, ex vivo, and in animal models.

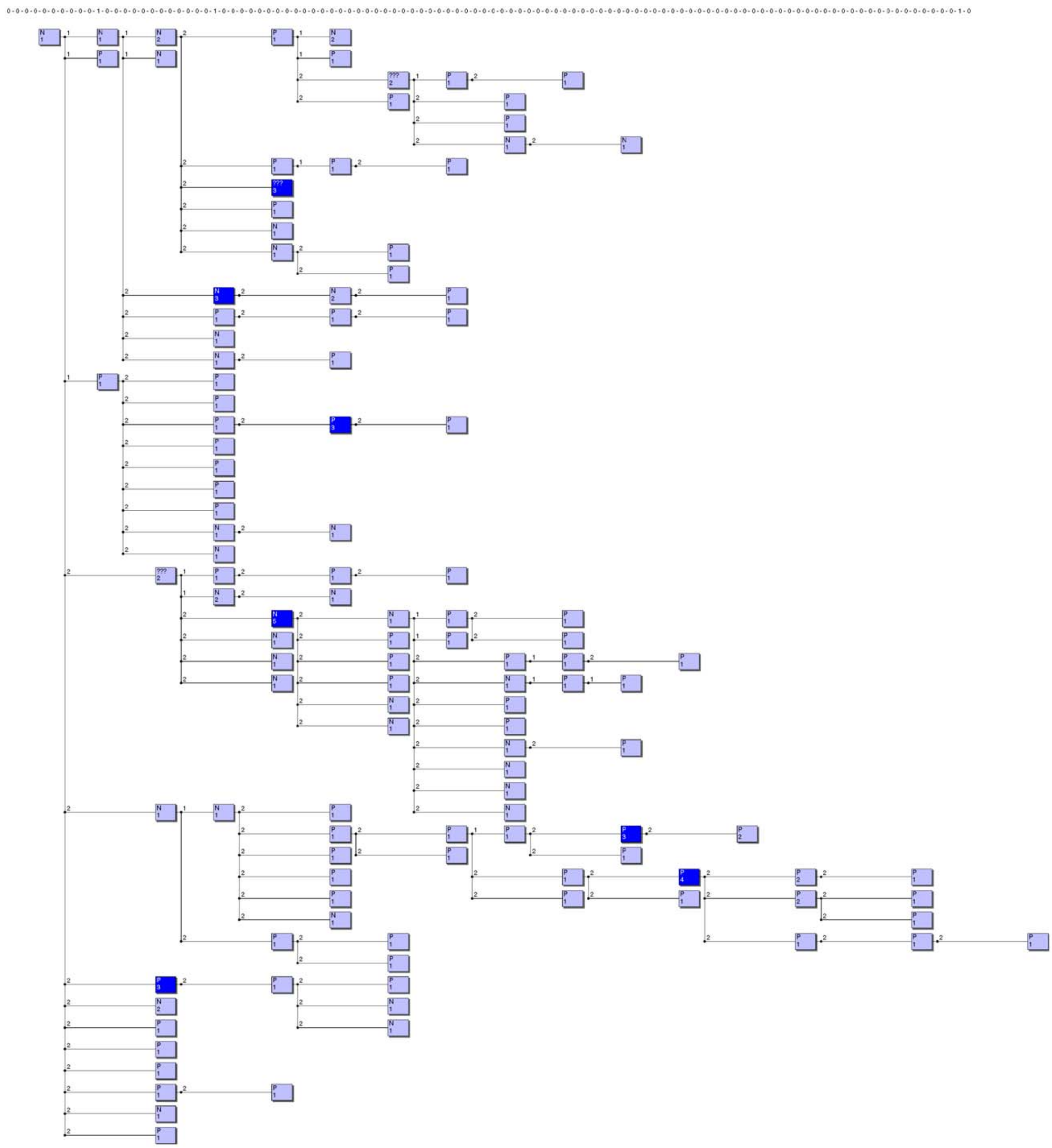

Fig. 4. Clonal complex 1; pathogenicity. The complex displays strain as pathogenic (P) or normal flora (N). Relations between strains are uni-directional starting from the founding genotype. Each rectangle shows strain(s) number(s) and shaded blue if more than 2 strains form a clone.

However, it is unlikely that virulence is a function of a single gene, especially under natural conditions. Likely, it is a multi-factorial trait [81] where multiple determinants share in adaptation (survivability and multiplication) of the organism to its niche and, consequently, in producing the pathological picture. For example, iron uptake machinery in many pathogenic prokaryotes [82] is not directly involved in producing the pathological picture, but its absence will be detrimental for the organism in iron-limiting conditions in vivo. Similarly, more than a single determinant contribute to production of *C. perfringens* gas gangrene [83,84]. Furthermore, virulence-associated genes are likely involved in general adaptability, fitness, and competitiveness in the niche [85]. Evidence of this comes from *Escherichia coli*, which exhibits a dual life style similar to that of *C. perfringens*, with virulence associated genes existing in commensal strains [86,87].

We used the recently developed MLVA scheme to investigate the evolution of virulence and population structure in *C. perfringens* [59] and incorporated seventy

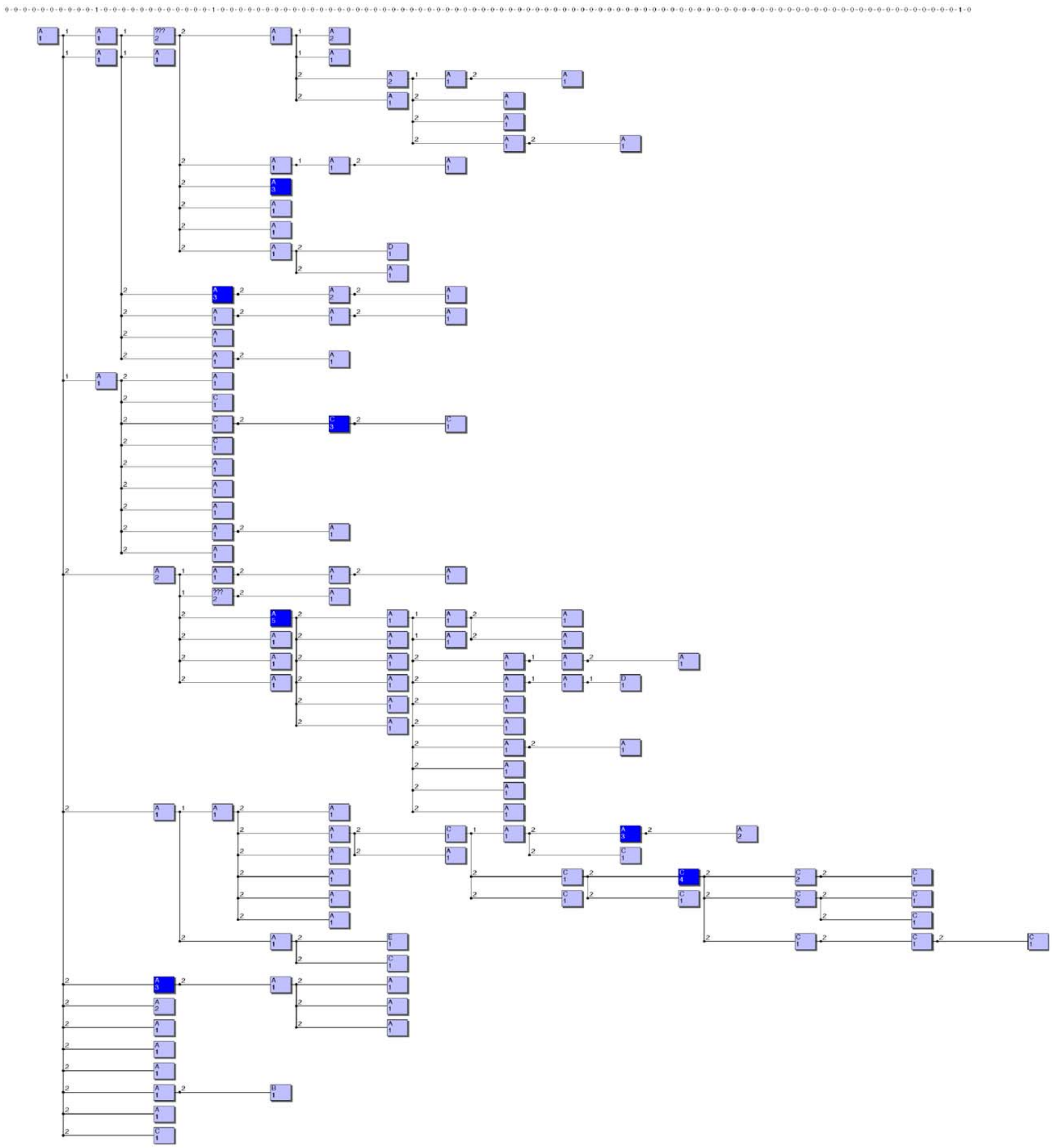

Fig. 5. Clonal complex 1; major toxin genes. The complex displays strain as major toxin genes. Relations between strains are uni-directional starting from the founding genotype. Each rectangle shows strain(s) number(s) and shaded blue if more than 2 strains form a clone.

five *C. perfringens* flora strains into the analysis. Consistent with what is known about this species, all the normal flora strains belonged to toxin type A. On the other hand, existence of *cpb2* in 45% of these strains argues for the possibility of its involvement in adaptation to the niche. However, the variation in carriage percentage among hosts possibly reflects the availability of the DNA donor or variation in selective pressure among different niches.

Unlike what might be expected from major-toxin typing data, strains of various toxin types do not have separate evolutionary histories; rather, the phylogenetic tree suggests that acquisition of *cpb*, *etx*, and *iap*/*ibp* is a relatively recent event. Moreover, the existence of strains of two different toxin types in the same clone supports this conclusion. Even in the case of type E strains, which show a significant degree of association between strains' phylogeny and toxin type, strains of other toxin types can be detected on the same cluster, suggesting again that this association is a recent event and could be due to epidemic clone expansion [65]. More supportive data come from the

MST, where *cpb*, *etx*, or *iap*/*ibp* acquisition/loss is evident. While this has been previously suggested [14,88], to our knowledge this is the first report which shows that this process occurs in natural population of *C. perfringens*.

Acquisition of these genes (*cpb*, *etx*, or *iap*/*ibp*) has been suggested to occur by lateral gene transfer (LGT) from other bacterial species [14], and the frequency of isolation of strains of these toxin types from certain host species might be a reflection of the availability of DNA donors in the respective host species. However, maintenance of these genes is essentially a function of the selective advantage that these gene(s) confer to strains carrying them in a specific niche during the disease process, especially since all of them are linked to mobile genetic elements and exist on plasmids [16,26,28]. In this case, maintaining selective pressure will ensure vertical inheritance of these toxin genes, and recovery from different host species (type D from sheep and goats, type C from swine and cattle) where in both niches the toxin gene is adaptive and enables the strain to compete and eventually produce the pathological lesion. Moreover, maintenance of selective pressure and vertical transmission can explain the unique clustering of type E strains and frequent episodes of type C clonal expansion seen on the NJ and MST tree. Additional evidence comes from reports where toxin type switching has been noticed in vitro [14,16,89] and that is essentially an example of lack of selective pressure.

A similar argument would explain the erratic distribution of other toxin genes that are carried on plasmids and/or linked to mobile genetic elements. In addition, the selective advantage conferred by some of these toxin genes probably changes between health and disease condition in the intestinal tract and this would explain the change in percentage of carriage of *cpb2* from 25% in healthy pigs, to 90% during type A porcine enteritis. Also, this would strengthen our contention that virulence, at least in this species, is not a function of a single gene.

Lack of association between strains phylogeny and host species or disease is evident on the NJ tree, suggesting the ability of a virulent strain to cause disease in different host species. More supportive data comes from the MST, where virulent strain from a specific host would be the ancestor of a pathogenic strain in a different host species. However, if this is the true scenario in the natural population, we should be able to find evidence that it works in both directions. Indeed, this is probably the situation, as, for example, JGS4095 (type A equine enterocolitis strain) is the founding genotype of the three poultry necrotic enteritis strains (JGS4143, 4141, 4135). Vice versa,

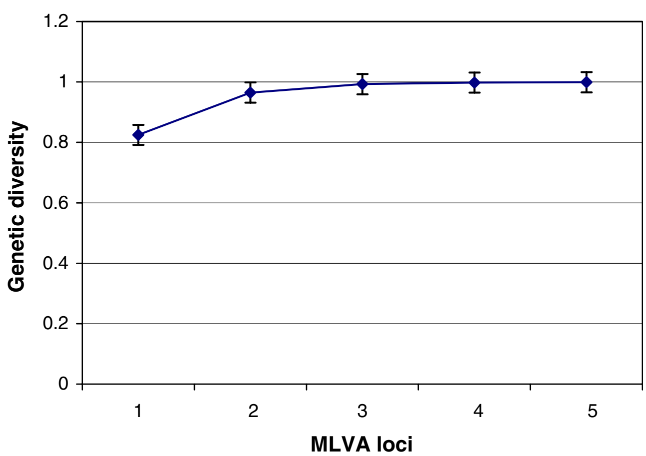

Fig. 6. *C. perfringens* genetic diversity using five MLVA loci. Genetic diversity of the population reached a plateau when five loci were included in the analysis. Error bars indicate standard error.

Table 3
McDonald–Kreitman 2 × 2 table

		Fixed	Polymorphic	
Plc	Synonymous	53	23	NI: 1.69, $P = 0.047$[a,*]
	Non-synonymous	225	165	
ColA	Synonymous	333	16	NI: 0.539, $P = 0.0692$[a]
	Non-synonymous	927	24	

[a] *G* value with William's correction.
[*] significant; NI, neutrality index.

Table 2
Population structure indices

Level	Number of genotypes	Frequency of the most frequent genotype	Diversity	Association indices		
				I_A	$\bar{r}_d$	P
All isolates	295	5	0.999	−0.651	−0.2493	<0.01*
Clade 1	5	2	0.904	0.386	0.130	0.140
Clade2-1	92	5	0.997	0.113	0.030	0.01*
Clade2-2-1	63	2	0.998	0.053	0.0139	0.18
Clade2-2-2-1	86	3	0.997	0.019	0.0048	0.360
Clade2-2-2-2	49	4	0.989	0.110	0.0279	0.060
Type C	33	4	0.985	0.558	0.1439	<0.01*
Type D	25	1	1.00	0.052	0.0146	0.290
Type E	21	2	0.995	0.2743	0.0723	<0.01*

I_A, index of association [65]; $\bar{r}_d$, index of association with correction [64]; *significant.

JGS4043, a poultry necrotic enteritis strain, is the founding genotype of JGS4157, a type A strain isolated from equine sudden death, and JGS1614, a type A equine clinical case.

Interestingly, was the phylogenetic and MST evidence that some normal flora strains from a certain host species are phylogenetically close to or founding genotypes of virulent strains from a different host species. This works in both directions; H-5-6, a porcine flora strain, is the founding genotype of JGS1796, a type A strain isolated from bovine enteritis. Vice versa, a porcine hemorrhagic enteritis type A strain (JGS1587) shares a single recent ancestor with bovine flora strain B-5. A similar transition to virulence following change of the niche is also evident in the case of *E. coli* O157:H7, which colonizes the lymphoid follicle-dense mucosa of the terminal rectum of healthy cattle [90] and ˜80% of cattle can harbor the organism [91–93]. However, if *E. coli* O157:H7 gains access to a human host, it can cause bloody diarrhea which progresses into hemolytic uremic syndrome in 5% of infected persons [94–97]. For both micro-organisms, this transition can be explained by variation in the degree of selective advantage that virulence determinants confer in different niches.

Lack of significant linkage disequilibrium is usually taken as an indication of a panmictic population. However, analysis of *C. perfringens* population revealed significant panmixia, and this is frequently interrupted by episodes of clonal expansion that in most cases correspond to disease processes (e.g., in case of types C and E strains). This indicates that recombination events are the major factor shaping *C. perfringens* population and would propose the molecular mechanism that explains the phylogenetic as well as MST evidence and the erratic distribution of chromosomally encoded virulence genes. It would also explain how genes/alleles could be transferred between strains with subsequent increase or decrease in virulence. Supportive evidence of homologous recombination comes from the observation that genes (*plc*, *pfoA*, *colA*, *nagH*) are located on variable regions of the chromosome, as determined by I-*Ceu*I genome mapping [16,17]. I-*Ceu*I recognizes 19 bp sequence in the RNA operons [98], and the probability of another similar restriction site just by point mutations is very low if it is not zero. Thus, the observed size variation can be explained by recombination, insertion, or deletion and all other things being equal, recombination is the simplest of these.

Genes associated with adaptation are likely subject to positive selection [99]. With continuous selective pressure, genic diversity at this locus (beneficial mutations) will be promoted, resulting in alleles that may confer different degrees of selective advantage in different niches. An alpha toxin gene with divergent DNA sequence (85% identity to strain 13 *plc*) has been recently characterized [100]. Testing whether this divergence is a result of molecular adaptation shows that this is likely the case. Extending the analysis to *colA* showed the two alleles to have mutations that are selectively neutral or nearly neutral. However, the results with *colA* are not conclusive, and availability of more sequences may result in detection of adaptive mechanisms at this locus.

Finally, the results presented here show that *C. perfringens* has a dynamic population where genetic determinants are swapped among strains. Also, it indicates that major toxin-typing, while very efficient, does not display virulence or virulence potential of type A strains including strains recovered from healthy animals. Availability of more genome sequences, use of comparative proteomics, and of animal models should provide more insight into the virulence of type A as well as other toxin types strains and help devise protective methods.

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