



Fine-Scale Structure Analysis Shows Epidemic Patterns of Clonal Complex 95, a Cosmopolitan *Escherichia coli* Lineage Responsible for Extraintestinal Infection

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ABSTRACT The *Escherichia coli* lineage known as clonal complex 95 (CC95) is a cosmopolitan human-associated lineage responsible for a significant fraction of extraintestinal infections of humans. Whole-genome sequence data of 200 CC95 strains from various origins enabled determination of the CC95 pangenome. The pangenome analysis revealed that strains of the complex could be assigned to one of five subgroups that vary in their serotype, extraintestinal virulence, virulence gene content, and antibiotic resistance gene profile. A total of 511 CC95 strains isolated from humans living in France, Australia, and the United States were screened for their subgroup membership using a PCR-based method. The CC95 subgroups are nonrandomly distributed with respect to their geographic origin. The relative frequency of the subgroups was shown to change through time, although the nature of the changes varies with continent. Strains of the subgroups are also nonrandomly distributed with respect to source of isolation (blood, urine, or feces) and host sex. Collectively, the evidence indicates that although strains belonging to CC95 may be cosmopolitan, human movement patterns have been insufficient to homogenize the distribution of the CC95 subgroups. Rather, the manner in which CC95 strains evolve appears to vary both spatially and temporally. Although CC95 strains appeared globally as pandemic, fine-scale structure analysis shows epidemic patterns of the CC95 subgroups. Furthermore, the observation that the relative frequency of CC95 subgroups at a single locality has changed over time indicates that the relative fitness of the subgroups has changed.

IMPORTANCE *Escherichia coli* clonal complex 95 represents a cosmopolitan, genetically diverse lineage, and the extensive substructure observed in this lineage is epidemiologically and clinically relevant. The frequency with which CC95 strains are responsible for extraintestinal infection appears to have been stable over the past 15 years. However, the different subgroups identified within this lineage have an epidemic structure depending on the host, sample, continent, and time. Thus, the evolution and spread of strains belonging to CC95 are very different from those of an-

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other cosmopolitan human-associated clonal complex, CC131, which has increased significantly in frequency as a cause of extraintestinal infection over the past 15 years due to the evolution and spread of two very closely related, nearly monomorphic lineages.

KEYWORDS clonal complex 95, *Escherichia coli*, epidemiology, population genetics

Many of the bacteria that cause disease in humans represent geographically widespread, human-specific, nearly monomorphic lineages within species that are much more genetically diverse (1). High-throughput sequencing technologies now allow investigation of the population structure and evolution of these pathogens (2–6). Such studies have revealed that these pathogens are relatively new, with their most recent common ancestor existing from 3,000 to 500 years ago. These studies have also revealed where some of these pathogens originated, how they have spread geographically, and, in some instances, how they have acquired resistance to antimicrobial agents.

Escherichia coli is a genetically diverse species that is primarily a commensal of the lower intestinal tract of mammals and, to a lesser extent, birds. However, particular lineages of *E. coli* are well-known intestinal pathogens that cause diarrhea, most notably the enterohemorrhagic strain O157:H7. Additionally, *E. coli* is the most common cause of urinary tract infection and a leading cause of bloodstream infection. Although *E. coli* isolates from extraintestinal sites in humans include diverse strains, a very significant fraction represent just a few lineages (7, 8). Indeed, the great majority belong to fewer than two dozen clonal complexes (CCs), namely, those represented by sequence type 10 (ST10 [phylogroup A]), STs 12, 14, 73, 95, 127, 131, 141, 144, and 372 (all phylogroup B2), and ST69 (phylogroup D). However, most extraintestinal isolates belong to just one of four well-defined CCs, CC73, -95, -131, and -69, which are geographically widespread, if not cosmopolitan (8). There is a well-developed understanding of the epidemiology and genetic structure of CC131 (9, 10), as members of the complex have, in recent years, become one of the most frequent causes of extraintestinal infection by *E. coli*. In contrast, little is known about members of CC95, -73, or -69.

The present study focuses on strains belonging to CC95, a cosmopolitan lineage that in previous studies has been responsible for, on average, 17% of extraintestinal infections in humans caused by *E. coli* (11–17). We used whole-genome sequence (WGS) data and PCR analysis to investigate a large collection of CC95 isolates from diverse time periods, locales, hosts, and contexts (i.e., commensal versus infection isolates). The results of this analysis reveal that the diversity to be found within this clonal complex varies geographically, temporally, and with the strain's source of isolation (e.g., fecal versus extraintestinal) and the sex of the host (for human isolates).

RESULTS

Distribution and abundance of CC95: host range of CC95. The continent of Australia is home to diverse vertebrate species. The human population is sparsely distributed (3.06 people km⁻²) and highly urbanized (89%). Europeans occupied Australia beginning in 1788, and since European settlement, levels of sanitation have been relatively high. Consequently, Australia represents an ideal location in which to determine the diversity of vertebrate species in which CC95 may be found.

Large-scale surveys of *E. coli* from a variety of Australian vertebrate hosts have shown that *E. coli* can be recovered from any vertebrate host group (18, 19). However, strains belonging to CC95 have not been detected in the feces of any sampled native mammal ($n = 1,063$), backyard poultry ($n = 278$), amphibian ($n = 106$), or fish ($n = 138$); likewise, a CC95 strain was recovered from only 1 of 447 reptiles and 1 of 1,312 native birds. Overall, the probability of detecting a CC95 strain in the feces of a nonhuman "native" vertebrate is less than 0.06%. CC95 strains were recovered from 5.6% of 306 Australian samples of commercial poultry meat (D. M. Gordon and B. Vangchhia, unpublished data).

Although members of CC95 are rare in nonhuman vertebrates, in humans, CC95 isolates are not only frequently observed, but their frequency appears to be temporally stable. Two large collections of *E. coli* strains collected from human feces, urine, and blood by the Microbiology Laboratory of the Canberra Hospital, Australia, in 2002 (20) and again in 2014 and 2015 using nearly identical methods were assigned to a phylogroup and screened for members of CC95 as described in Materials and Methods. Although the frequency of phylogroup B2 strains was highest among urine isolates and lowest among fecal isolates, with blood isolates intermediate, there was no change in the relative abundance of phylogroup B2 strains between 2002 and 2014, irrespective of the isolate's source (feces, urine, or blood) (nominal logistic regression: year, $P = 0.98$; source, $P < 0.001$; year-source interaction, $P = 0.86$) (Fig. 1A). Among the B2 strains, the CC95 strains represented 19% of the isolates, but here again, there was no significant change in the frequency of CC95 isolates between 2002 and 2014 and no difference in the frequency of CC95 isolates with respect to the isolate's source (nominal logistic regression: year, $P = 0.07$; source, $P = 0.26$; year-source interaction, $P = 0.67$) (Fig. 1B).

CC95 pangenome analysis. (i) The pangenome. There were a total of 17,603 genes among 200 CC95 strains recovered from feces and extraintestinal sites at a variety of geographic locations. The core genome (present in $\geq 99\%$ of strains) consisted of 3,134 genes: 534 genes were found in from 95% to $< 99\%$ of strains, 1,196 were found in 30% to $< 95\%$ of strains, and 11,934 were found in fewer than 15% of the strains. Over 5,400 genes were found in only a single isolate, and 2,210 genes were found in all 200 isolates, with the balance of the genes being present in > 1 or < 200 isolates, giving rise to a U-shaped distribution for the number of isolates in which a gene is present (Fig. 2).

(ii) Core genome variation. The phylogeny inferred for these 200 CC95 isolates revealed, as expected, significant structure within CC95, with the observed clusters correlating largely with a strain's serotype (Fig. 3). Five main clusters were evident. The cluster of strains with an O1:H7 or an O2a:H7 serotype was designated subgroup A. The cluster of strains with an O18:H7 serotype, including UT189, was designated subgroup B. The cluster of O1:H7 strains, which also included O25b:H4 and O2a:H7 strains, was designated subgroup C. The cluster of strains that included those with O45a:H7, O1:H7, and O2a:H4 serotypes was designated subgroup D. The cluster of strains with an O2a:H4 serotype was designated subgroup E. Additionally, 9 (5%) of the 200 strains examined did not fall into one of these five subgroups, and 4 of these strains with the H5 antigen and *fimH* allele 15 were distinct from the other CC95 strains (Fig. 3). Within four of the five CC95 subgroups, strains that shared the same serotype also tended to share the same *fimH* allele (21). The sole exception was the subgroup B strains, which despite all being O18:H7 exhibit four different *fimH* alleles (Fig. 3).

Subgroup D comprised strains of diverse serotypes. However, there seemed to be little value in further subdividing subgroup D, as all of the subgroup D strains were separated from the other subgroups by long branches. Additionally, a practical reason for not subdividing subgroup D is that although CC95 strains are relatively common, they will never represent a large fraction of isolates in any strain collection, and smaller sample sizes mean less statistical power.

(iii) Variable gene content. Analysis of variable genes showed that the substructure revealed by the core genome phylogeny was reflected in the variable gene content of the strains (Fig. 4). Subgroups A, B, and C had variable gene contents that were distinct from those of the other phylogroups. The subgroup E strains were an exception, since despite being tightly clustered and distinct according to the core genome, they showed some variable gene content overlap with subgroup D strains.

Multiple genes within the variable genome have been identified as potentially enhancing a strain's propensity to cause extraintestinal disease or colonize a host (22, 23). *In silico* analysis of 40 such virulence factors among the 200 CC95 isolates showed that *afdA*, as well as *focG*, *lpfA*, and *terC*, is absent from all strains (see Table S1 in the supplemental material). In contrast, all CC95 strains have *fimH*, *fyuA*, and *ompT*. Be-

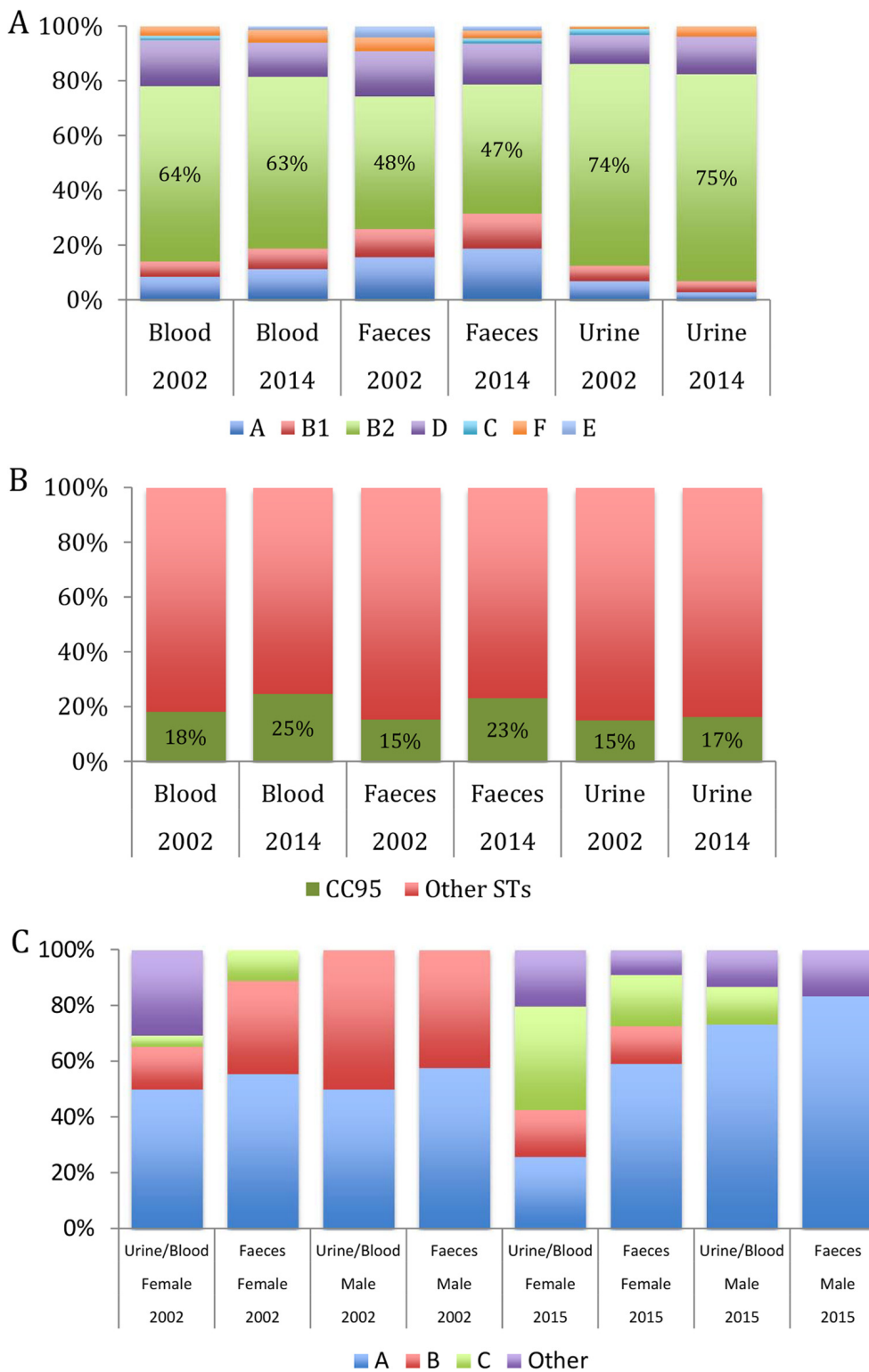


FIG 1 Relative frequency of phylogroups, CC95 isolates, and CC95 subgroups among *E. coli* strains recovered from faeces, urine, or blood samples taken from people living in Canberra in 2002 and 2014. (A) Relative frequency of phylogroups among *E. coli* isolates; (B) relative frequency of CC95 strains among B2 strains; (C) Relative frequency of CC95 subgroups among CC95 strains.

tween these extremes, less than 5% of CC95 strains have the gene *sfa*, *iha*, or *tsh* or harbor the plasmid encoding microcin B17 or colicins B and M, whereas more than 95% have the virulence genes *neuC*, *sitA*, *usp*, and *vat*. Other virulence genes have midrange prevalence values.

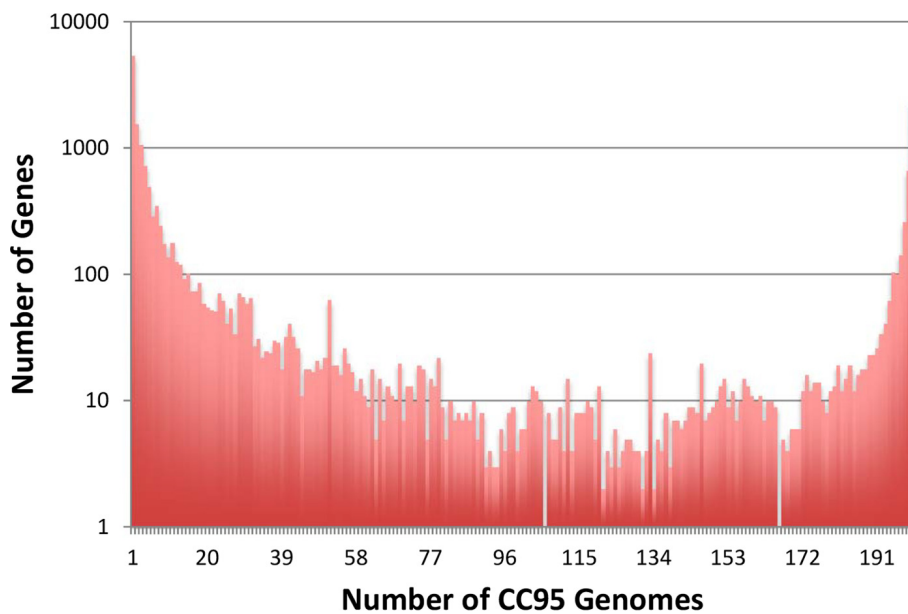


FIG 2 Distribution of the genes in the pangenome of *E. coli* CC95. The number of genes found in 1 to 200 strains, respectively, is shown. All genomes were annotated using Prokka, and the pangenome was determined using ROARY.

As with serotype, the presence of many virulence genes varied in relation to the subgroup membership of the CC95 strain (Table 1). Overall, subgroup A strains possessed the colibactin gene cluster, the self-adhesion locus antigen 43, and the toxins *tcpC* and *senB*. Subgroup B strains also have the colibactin locus, but in addition possessed the invasion of brain epithelium gene *ibeA* and were likely to have the invasion determinant *tia* and the toxin determinants *cnf1*, *hylD*, and *cdiA*. Relative to strains belonging to subgroups A and B, strains belonging to subgroups C, D, and E had

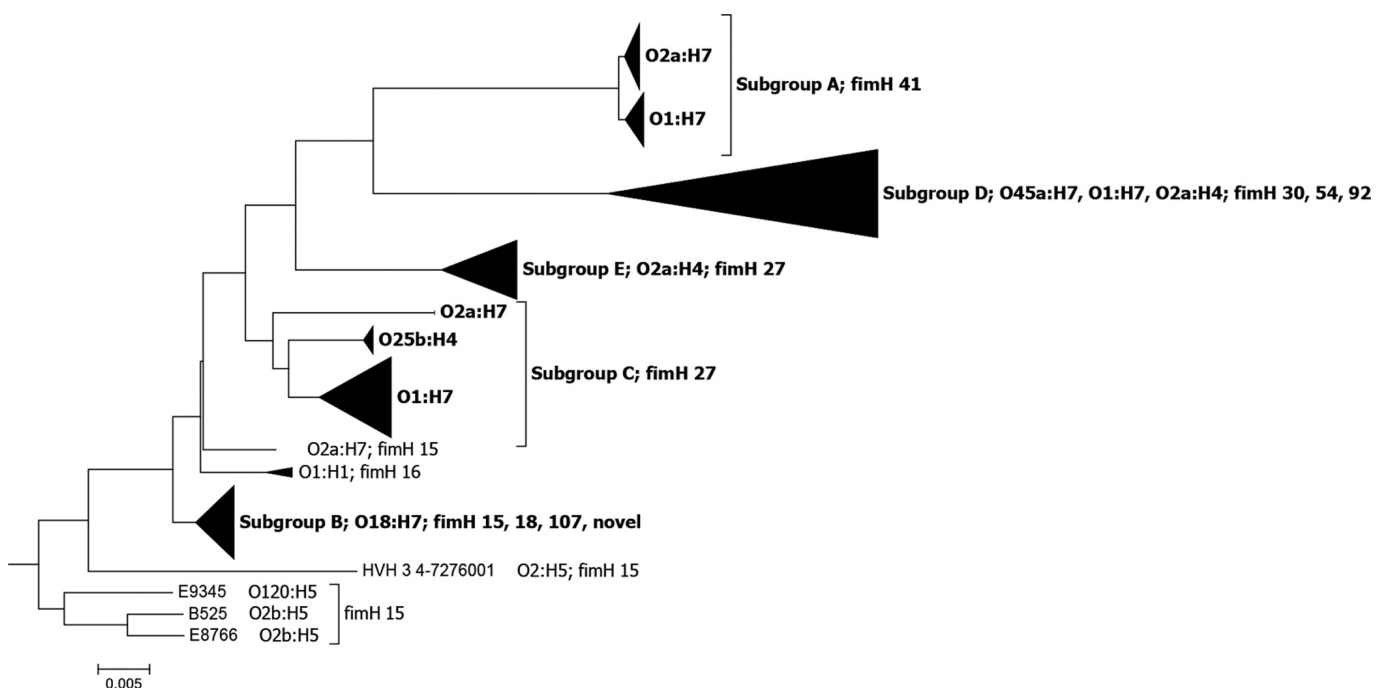


FIG 3 Substructure of 200 *E. coli* CC95 strains. Group B2 strain ED1a was used as an outgroup (not shown). The single nucleotide polymorphisms (SNPs) were detected using the Harvest Suite (43) of tools and ED1a as the reference strain. Gubbins (53) was used to infer recombination events, and recombinant sites were removed. A maximum likelihood tree was inferred with a general time-reversible (GTR) model of evolution using MEGA 6.0 (54).

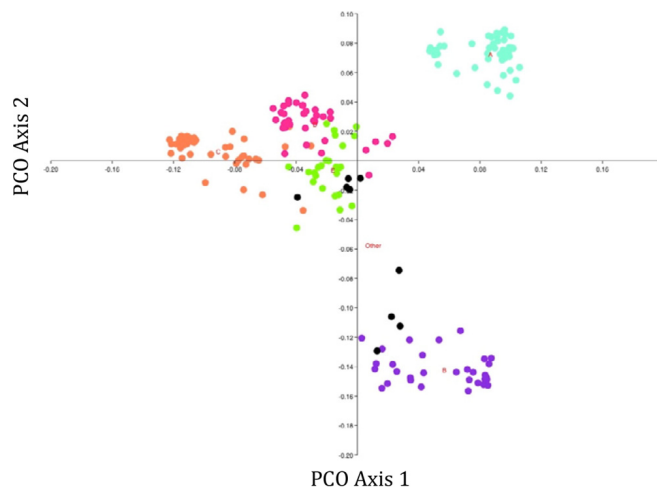


FIG 4 Similarity among *E. coli* CC95 isolates based on the variable genome. Genes present in all genomes or in a single strain were eliminated. Among-strain similarity was quantified using a Jaccard metric, and the dimensions of the matrix were reduced using principal-coordinate (PCO) analysis as implemented in PAST. Turquoise denotes subgroup A strains, purple B strains, orange C strains, pink D strains, green E strains, and black unassigned strains.

more similar extraintestinal virulence factor profiles, but differed from subgroup A and B strains in having the plasmid-encoded bacteriocins colicin Ia and microcin V. Subgroup C, D, and E strains were also very likely to possess the iron-uptake-related genes, *ireA*, *iroN*, and *iucAC*. Subgroup C strains typically hosted a colicin E1 plasmid, while subgroup D and E strains did not. Most subgroup D strains possessed antigen 43, while subgroup C and E strains did not.

(iv) Antibiotic resistance determinants. None of the 200 CC95 isolates had the chromosomal mutations in *gyrA* or *parC* associated with fluoroquinolone resistance. To investigate the extent to which the presence of resistant determinants that are typically plasmid associated varied among CC95 subgroups, the analysis was restricted to the 83 Australian strains from humans for which whole-genome sequence (WGS) data were

TABLE 1 Distribution of putative virulence factors among strains belonging to the five CC95 subgroups

Gene	% in CC95 subgroup:					P value ^a
	A (n = 51)	B (n = 31)	C (n = 48)	D (n = 38)	E (n = 25)	
<i>clbB</i>	100	100	4	0	44	<0.001
<i>cnf1</i>	0	58	0	0	0	<0.001
Colicin E1 gene	0	0	81	8	44	<0.001
Colicin Ia gene	24	23	83	68	52	<0.001
<i>etsC</i>	24	39	87	74	36	<0.001
<i>hylD</i>	0	58	0	13	0	<0.001
<i>ibeA</i>	0	100	2	0	0	<0.001
<i>ireA</i>	90	13	100	95	100	<0.001
<i>iroN</i>	24	94	87	87	60	<0.001
<i>iucC</i>	24	35	90	86	84	<0.001
<i>iutA</i>	25	35	90	84	84	<0.001
Microcin V gene	24	29	81	74	52	<0.001
<i>papC</i>	90	55	100	95	96	<0.001
<i>tcpC</i>	90	0	0	3	0	<0.001
<i>traT</i>	92	84	94	87	88	0.607
Antigen 43 gene	94	87	4	95	4	<0.001
<i>cdiA</i>	0	58	0	0	0	<0.001
Microcin H47 gene	22	29	71	48	70	<0.001
<i>tia</i>	0	74	2	0	0	<0.001
<i>cdtB</i>	0	29	2	5	4	<0.001
<i>senB</i>	69	42	2	0	4	<0.001

^aContingency table χ^2 testing if gene is nonuniformly distributed among CC95 subgroups.

TABLE 2 Frequency of plasmid-borne antibiotic resistance determinants among CC95 isolates from humans living in Canberra, Australia, with respect to their subgroup membership and serotype

Variant	% of subgroup isolates with determinant						P value ^a
	A, O1:H7 (n = 11)	A, O2:H7 (n = 18)	B, O18:H7 (n = 12)	C, O1:H7 (n = 18)	C, O25b:H4 (n = 8)	E, O2:H4 (n = 15)	
<i>aadA1</i>	0	57.9	0	0	50.0	0	<0.001
<i>bla</i> _{TEM-1B}	9.1	52.6	41.7	5.6	62.5	46.7	0.005
<i>bla</i> _{TEM-1C}	27.3	21.1	8.3	5.6	12.5	6.7	0.48
<i>strA</i>	9.1	21.1	0	16.7	12.5	26.7	0.29
<i>strB</i>	0	0	0	11.1	0	26.7	0.03
<i>sul1</i>	0	63.2	0	16.7	25.0	20.0	<0.001
<i>sul2</i>	9.1	21.1	0	16.7	12.5	26.7	0.49
<i>tetB</i>	0	0	0	88.9	25.0	0	<0.001

^aContingency table χ^2 testing if gene is nonuniformly distributed among CC95 subgroups.

available. Preliminary analysis indicated that presence of particular resistance determinants varied in relation to both serotype and CC95 subgroup. Consequently, for statistical comparisons the subgroup A strains were split into serotypes O1:H7 and O2a:H4, and the subgroup C strains were split into serotypes O1:H7 and O25b:H4. Subgroup D and the unassigned strains were excluded due to small sample sizes. Statistical analysis of individual plasmid-borne antibiotic resistance determinants was restricted to those determinants observed 6 or more times (the number of subgroup/serotype categories).

Overall, plasmid-borne resistant determinants were uncommon among the 83 CC95 strains, with the numbers of detected determinants per strain (shown as a percentage of 83 strains in parentheses) being 0 (31.3%), 1 (25.3%), 2 (4.8%), 3 (18.1%), and ≥ 4 (20.5%). The number of plasmid-borne determinants varied significantly with the serotype/subgroup membership of a strain (Kruskal-Wallis test, $\chi^2 = 19.6$, $P = 0.0015$). Subgroup A O1:H7 and subgroup B O18:H7 strains carried, on average, less than 1 plasmid-encoded resistance determinant each, subgroup A O2a:H7, subgroup C O1:H7, and subgroup E O2a:H4 strains typically carried 2 such determinants, and subgroup C O25b:H4 strains carried more than 4 such determinants (Table 2). The full plasmid-encoded antibiotic resistance gene profiles of all CC95 study isolates are presented in Table S1.

Intrinsic extraintestinal virulence of CC95. Phylogroup B2 strains generally are known to be highly virulent in mouse models of extraintestinal infection (24). The virulence of 58 CC95 strains isolated from Australia, France, and the United States, which included representatives of all five CC95 subgroups, was assayed using the mouse sepsis model. Consistent with their expected high virulence, 82% of strains killed all of the mice challenged with the strain. The strains' subgroup membership explained some of the variation in ability to kill 100% of tested mice (contingency table analysis: likelihood ratio, $\chi^2 = 12.27$, $P = 0.002$). Killing of all tested mice was observed for all subgroup D strains ($n = 12$), 94% of subgroup B strains ($n = 18$), and 62% of subgroup A strains ($n = 24$). Strains belonging to CC95 subgroups E and C also killed 100% of mice tested, but were not included in the preceding analysis, as only a total of 6 strains from these two subgroups were tested.

Multiplex PCR for CC95 subgroup assignment. The pangenome analysis revealed which genes were present in all members of a subgroup, but rare or absent in the other subgroups. This allowed a multiplex PCR screening tool to be developed for subgroup identification (see Materials and Methods for details and Fig. 5 for an example of the assay). Application of the multiplex method to those strains from Australia and France for which whole-genome sequence data were available showed that the method correctly assigned 92% of the strains to the appropriate subgroup (Table 3).

Epidemiology of CC95 subgroups. The availability of simple PCR-based screening methods for determination of the CC95 subgroup membership of CC95 strains allowed collections of CC95 isolates from Australia, France, and the United States to be screened for the relative abundance of the CC95 subgroups. This in turn allowed an analysis of factors influencing the relative abundance of CC95 subgroups.

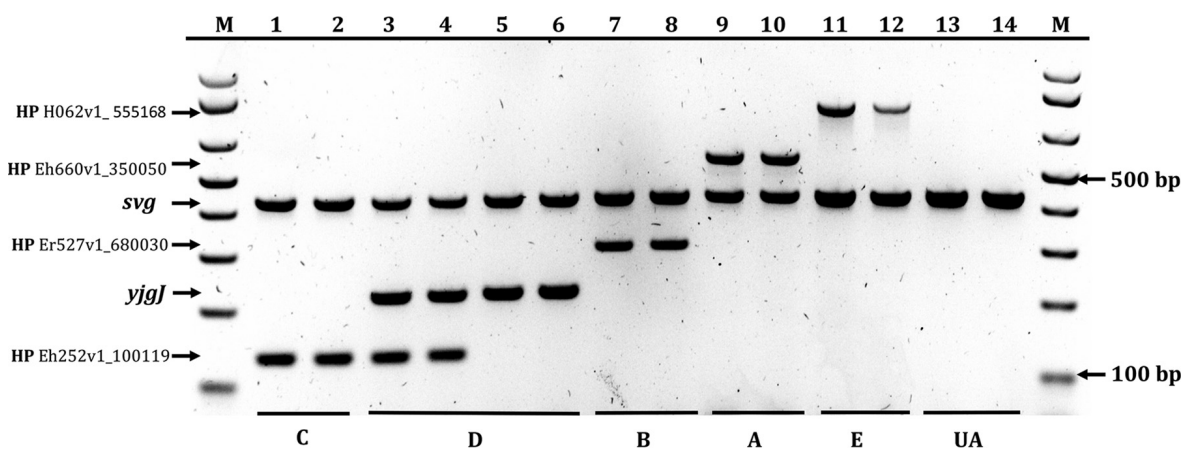


FIG 5 *E. coli* CC95 subgrouping multiplex PCR. The *svg* band corresponds to the CC95 control band (positive on all strains), whereas the HP H062v1_555168, HP Eh660v1_350050, HP Er527v1_680030, *yjgJ*, and HP Eh252v1_100119 bands correspond to the amplification of subgroup E-, A-, B-, D-, and C-specific genes, respectively. UA, unassignable. Lanes: 1, H252 (O1:H7); 2, 004-008 (O25b:H4); 3, B5H8 (O1:H7); 4, B118 (O2a:H4) (note the amplification of the subgroup C-specific gene in addition to the subgroup D-specific gene); 5, S88 (O45a:H7); 6, 034-018 (O1:H7); 7, UT189 (O18:H7); 8, 003-007 (O18:H7); 9, H660 (O1:H7); 10, B6H13 (O2a:H7); 11, 0032-001 (O2a:H4); 12, H062 (O2a:H4); 13, H151 (O1:H1); 14, NILS21 (O2b:H5). M, molecular size marker (1-kb Plus DNA ladder; Invitrogen).

(i) Distribution of CC95 subgroups in Australia. Two large collections of *E. coli* isolates collected from human feces, urine, and blood by the Microbiology Laboratory of the Canberra Hospital, Australia, in 2002 (20) and again in 2014 and 2015 yielded 172 CC95 strains, distributed by subgroup as follows: 48% A, 18% B, 18% C, 3% D, 9% E, and 4% indeterminate. Host age and sex were known for 147 CC95 strains, in addition to the strain's source and year of isolation. Strains were classified as having either a fecal or extraintestinal (blood or urine) source. Strains of subgroups D and E and unassigned strains were uncommon and so were pooled (Fig. 1C). Nominal logistic regression analysis revealed that the relative abundance of CC95 subgroups varied significantly with strain source, year of isolation, and host sex, but not host age (isolate source, $P = 0.04$; year of isolation, $P < 0.001$; host sex, $P = 0.01$; year of isolation-host sex interaction, $P = 0.003$). (Backwards elimination was used to eliminate nonsignificant terms, and only statistically significant terms are presented.)

The underlying prevalence values were inspected to determine the basis for these results. For the 2002 isolates, subgroup B strains were isolated more frequently from extraintestinal infection and from males than females. For the 2015 isolates, subgroup

TABLE 3 Assessment of the ability of the subgroup CC95-specific genes together with serotype to correctly assign CC95 strains to their correct subgroup

Phylogeny assignment	Multiplex PCR assignment(s)	PCR serotype	No. of isolates ^a
A		O2a:H7	5
A	A	O1:H-	3
A	A	O1:H7	9
A	A	O2a:H7	16
B	B	O18:H7	19
C		O1:H7	1
C	C	O1:H7	32
C	C	O25b:H4	9
D	D	O1:H7	6
D	D	O2a:H4	2
D	D	O45:H7	2
D	D and C	O1:H7	3
D	D and C	O2a:H4	1
E	E	O2a:H4	16
Other		O1:H1	3
Other	C	O2a:H7	1
Other	A	O120:H5	1
Other	A	O2b:H5	2

^aOnly those strains sequenced as part of this study have been included.

A strains were more frequent among fecal isolates than extraintestinal isolates, subgroup B strains were confined to females, and subgroup C strains were more common among females than males. In comparison with the 2002 isolates, the 2015 isolates had a lower frequency of subgroup B and a higher frequency of subgroup C, the latter reflecting an increase in the frequency of both the O1:H7 and O25b:H4 serotypes.

(ii) Distribution of CC95 subgroups in France. Four collections of *E. coli* isolates from France (two of blood isolates and two of fecal isolates) were screened for CC95 strains, and the subgroup membership of the identified CC95 strains was determined. The blood isolates were collected in 2005 (25) and in 2005 to 2007 (13), while the fecal isolates were collected in 2000 (26) and 2010 (27). The 208 CC95 strains detected in these collections were distributed by subgroup as follows: 4% A, 12% B, 4% C, 69% D, and 9% E, and 1 strain could not be assigned to a subgroup.

Although the two sets of blood isolates were collected at similar times, they were taken from either adults or children, while the two sets of fecal isolates were collected 10 years apart. Thus, sample sizes were insufficient to permit an analysis that incorporated the effects of isolate source, host age, and year. However, a significant difference in the relative abundance of CC95 subgroups was detected when comparing the CC95 fecal and blood isolates (contingency table analysis: likelihood ratio, $\chi^2 = 12.46$, $P = 0.01$). Among the 174 CC95 blood isolates, 4% were subgroup A, 10% B, 3% C, 74% D, and 16% E, and 1 isolate was unassigned. In contrast, among the 33 CC95 fecal isolates, 6% were A, 26% B, 12% C, 47% D, and 9% E. Whereas the CC95 blood strains belonged overwhelmingly to subgroup D, the fecal strains, although also most likely to belong to subgroup D, also frequently belonged subgroup B.

In these collections, host age and sex were known for 147 CC95 strains isolated from blood. The relative abundance of CC95 subgroups varied with host sex but not with host age (nominal logistic regression: age, $\chi^2 = 5.06$, $P = 0.28$; sex, $\chi^2 = 17.55$, $P = 0.0015$; age-sex interaction, $\chi^2 = 6.15$, $P = 0.188$). Among the 114 CC95 blood isolates recovered from females, 3% were A, 6% B, 3% C, 77% D, and 11% E. In contrast, among the 33 blood isolates from males, 6% were A, 30% B, 0% C, 55% D, and 9% E. Although subgroup D isolates were the most likely subgroup to be recovered from females, subgroup D isolates were less likely to be observed among blood isolates from males and subgroup B isolates were more frequent.

(iii) Distribution of CC95 subgroups in the United States. Seven collections of *E. coli* strains from the United States acquired between 1981 and 2015 from human feces, blood, urine, and other extraintestinal sites and diverse localities (22, 28–32), were screened for CC95, and the subgroup membership of the identified CC95 strains was determined. The 146 detected CC95 strains were distributed by subgroup as follows: 65% A, 27% B, 3% D, and 4% E. None represented subgroup C.

A comparison of CC95 strains from blood and urine identified a significant source effect on the relative abundance of CC95 subgroups (contingency table analysis: likelihood ratio, $\chi^2 = 6.46$, $P > \chi^2 = 0.039$). The blood isolates belonged mainly to subgroup A (70%), with only a small minority from subgroup B (13%) or other backgrounds (17%). In contrast, although the urine isolates likewise belonged mainly to subgroup A (62%), 33% belonged to subgroup B, and only 5% represented other backgrounds.

The 104 urine isolates were collected over 27 years and exhibited a significant change in relative subgroup abundance over this period (nominal logistic regression: year of isolation, $\chi^2 = 5.06$, $P = 0.28$; sex, $\chi^2 = 17.55$, $P = 0.0015$; age-sex interaction, $\chi^2 = 15.09$, $P < 0.001$). Specifically, subgroup A declined in relative frequency, with a concomitant increase in the other subgroups.

(iv) Among-country subgroup frequency comparisons. The relative abundance of CC95 subgroups varies with the strain's source of isolation (blood, urine, or feces) (Fig. 1C), and the available CC95 data are quite biased among countries with respect to isolate source. Therefore, comparisons of the relative abundance of different subgroups

TABLE 4 Relative abundance of CC95 subgroups isolated from the same source in different countries

Source	Country	n	% of isolates of subgroup:					P value ^a
			A	B	C	D	E	
Urine	Australia	41	41	14	30	0	11	<0.001
	United States	99	66	29	0	2	3	
Blood	Australia	23	33	11	33	0	14	<0.001
	France	173	4	10	3	74	9	
Feces	Australia	27	64	11	14	4	7	<0.001
	France	34	6	26	12	47	9	

^aContingency table χ^2 testing if CC95 subgroups are nonuniformly distributed with respect to source.

among CC95 strains from Australia, France, and the United States were restricted to strains from the same sources.

In these specimen-type-stratified comparisons, among the urine isolates, those from the United States were predominantly from subgroups A and B, while those from Australia were predominantly from subgroups A and C (Table 4). Among the blood isolates, those from France were predominantly from subgroup D, whereas none of those from Australia were from subgroup D. Among the fecal isolates, those from France were mostly from subgroup D, but subgroup B isolates were also quite common, whereas those from Australia were mostly from subgroup A.

Virulence factor data obtained through PCR screening were available for many of the non-Australian CC95 strains as well as the Australian strains. However, given the very nonrandom distribution of CC95 subgroups among countries, there were insufficient sample sizes to compare strains belonging to all subgroups among all countries. Therefore, a comparison of the distributions of virulence factors was restricted to CC95 subgroup B (Table 5). These analyses revealed that the virulence trait profile of subgroup B CC95 isolates varied with the country of origin.

Of the observed geographic differences in virulence trait profiles, the most striking related to the *pap* operon. Specifically, the *pap* operon was absent in most isolates from France but present in most isolates from Australia and the United States, whereas among *pap*-positive isolates, all of those from France and the United States had *papG* allele III, while those from Australia had *papG* allele II. As for other virulence factors, the salmochelin locus (*iroN*) was less frequent among isolates from Australia than those from France or the United States, the aerobactin locus (*iut*) was common among isolates from France, but absent from most isolates from the United States, and cytotoxic necrotizing factor 1 (*cnf1*) was uncommon among isolates from France, but present in almost all isolates from the United States.

DISCUSSION

E. coli CC95 has a cosmopolitan distribution, as it has been recovered from every continent, including Antarctica. This clonal complex has a narrow host range—one seemingly restricted to humans and human-associated birds and mammals, such as commercial poultry and companion animals. Although data are limited, the available

TABLE 5 Frequency of specific virulence traits in CC95 subgroup B isolates from different countries

Virulence trait	% of isolates positive from:			P value ^a
	Australia (n = 12)	France (n = 25)	United States (n = 21)	
<i>papC</i>	67	4	71	<0.001
<i>iroN</i>	67	100	95	0.005
<i>iut</i>	33	80	5	<0.001
<i>cnf1</i>	58	8	95	<0.001

^aContingency table χ^2 testing if gene is nonuniformly distributed among CC95 subgroups.

evidence indicates that CC95 strains are capable of persisting in the human gut for extended periods, as has been observed for other B2 strains, as residence times of 2 to 4 years have been observed (33). Strains of the complex are significant extraintestinal pathogens of humans and globally appear to account for about 17% of infections caused by *E. coli*.

The pangenome of CC95 was estimated to be in excess of 17,000 genes, a value similar to that found in a collection of 20 strains representative of *E. coli* as a whole (34). The distribution of these genes among genomes was also very similar to that reported by Touchon et al. (34); about 21% of genes were present in >95% of the isolates, while 68% were present in <15% of isolates.

The existence of genetic substructure in CC95 has long been recognized (35, 36), and based on variation in the core genome, it appears that the great majority of ST95 isolates belong to one of five subgroups. The subgroups are nonrandomly distributed geographically, with subgroup D strains being encountered more commonly in Europe than in Australia or North America. Subgroup C strains with an O1:H7 serotype may be endemic to Australia, as they have not been observed in other parts of the world. The distribution of extraintestinal virulence traits also varies among subgroups and likewise in relation to geographic origin. These geographic differences extend to different alleles of a particular gene, as noted for *papG* allele III, which is far more likely to be observed among CC95 isolates from the United States than those from Australia.

The frequency of CC95 in human populations would be expected to change if the frequency of phylogroup B2 strains changed, as has been the case in France over the past 30 years (27). In the Australian *E. coli* collections, however, there was no change in the frequency of phylogroup B2 strains between 2002 and 2014, irrespective of a strain's origin (feces, urine, or blood) (Fig. 1A). Similarly, there was no change in the frequency of CC95 strains in the Australian collections with respect to either sample date or a strain's origin (Fig. 1B). Similarly, Day et al. (37), in examining the clonal composition of *E. coli* strains causing bacteremia in the United Kingdom and Ireland between 2001 and 2010, found that CC95 strains were responsible for 11.3% of bacteremia cases overall, with no real change in frequency over time.

In stark contrast to the apparent temporal stability within *E. coli* of the relative abundance of CC95 at the clonal complex level is the variability within CC95 of the relative abundance of the various CC95 subgroups between 2002 and 2104 in Australia (Fig. 1C) and between 2011 and 2015 in the United States. However, the natures of the changes on the two continents were different. In Australia, subgroup B isolates decline in frequency, while subgroup C isolates became more common, whereas in the United States, subgroup A declined in frequency and the frequency of subgroup B was unchanged.

The substructure that exists in CC95 is epidemiologically relevant as, depending on geographic location, strains of some subgroups are more likely to cause septicemia than urinary tract infection or are more likely to be recovered from feces than from extraintestinal sites. Members of some subgroups also appear to be less likely to be recovered from males or more likely to be recovered from extraintestinal sites in females. Additional support for the epidemiological relevance of the subgroups comes from results from the mouse sepsis model, which reflects a strain's extraintestinal virulence. These results indicate that although most CC95 strains are highly virulent, some are not, and that some of this variation in virulence is explained by a strain's subgroup membership.

Collectively, the available evidence indicates that although strains belonging to CC95 may be cosmopolitan, human movement patterns have been insufficient to homogenize the distribution of the CC95 subgroups. Rather, the manner in which CC95 strains evolve appears to vary both spatially and temporally. The observation that the relative frequency of CC95 subgroups at a single locality has changed over time indicates that the relative fitness of the subgroups has changed, although whether such changes are relative to other members of the clonal complex or to other strains of *E. coli* is unknown. Also unknown is the extent to which these apparent changes in relative

fitness are a consequence of direct competition between members of the different subgroups and of how the different subgroups respond to changes in the host/external environment. Stochastic effects undoubtedly also play a role, as might be the case for the dominance of *papG* allele III among subgroup B strains in North America.

The ST131 clonal complex (CC131) is another very common human-associated *E. coli* lineage. However, few similarities are apparent between the evolution of CC131 and CC95. CC131 is a genetically more diverse clonal complex than CC95, but much of the success of CC131 is due to the evolution and spread of two very closely related, virtually monomorphic, fluoroquinolone-resistant lineages with an O25b:H4 serotype, known as H30R/C1 and H30Rx/C2 (9, 10). These lineages were virtually unknown prior to the turn of the century but have now spread worldwide and are currently the *E. coli* lineages most likely to be responsible for extraintestinal infection (38). Although a number of hypotheses have been proposed to explain the success of these ST131 lineages, such as enhanced virulence and antibiotic resistance, none have strong empirical support.

Collectively, the evidence indicates that although CC95 may be a temporally stable pandemic lineage of *E. coli* that is often responsible for extraintestinal disease in humans, its evolution bears little resemblance to that of other pandemic pathogenic lineages, such as *Salmonella enterica* serovar Typhi, *Shigella sonnei*, or even *E. coli* CC131. Rather, the evolution of CC95 is shaped by local conditions operating on at least a continental scale and over relatively short time frames. Moreover, the differences among the various CC95 subgroups are epidemiologically relevant. The analytical approaches that have been used so successfully to investigate the evolution and spread of other human pathogens, such as *Yersinia pestis* and *Salmonella enterica* serovar Agona (39), are unlikely to be as successful for CC95 strains, given the extent of spatial and temporal variation observed in this complex.

The pangenome analysis revealed that the genetic structure of *Escherichia coli* is fractal in nature. At the species level, *E. coli* exhibits considerable genetic structure (phylogroups), as discerned by examining the phylogenetic relationships among strains based on genes of the core genome. This substructure is reflected also in the similarity analyses based on the species' variable gene content (40). The distribution of genes is such that a minority of genes of the pangenome are present in all strains, while most genes in the pangenome are present in just one or a few strains. Notably, the same patterns are observed among the strains within a single clonal complex. CC95 exhibits substructure in its core genome, and this substructure is reflected in the variable genome of CC95, with most genes present in few isolates and a minority of genes present in all isolates. The results of this analysis of CC95 pangenome and of the analysis by Touchon et al. (34) of the *E. coli* pangenome cannot be compared directly due to differences in methodology. However, the size of the pangenome of CC95 is certainly close to that of the species as a whole. It is remarkable that the same pangenome can reflect the core genome structure observed for both the whole species and a clonal complex within the species. Further studies are required to elucidate the evolutionary processes that might lead to the observed patterns.

MATERIALS AND METHODS

Determining the CC95 pangenome. The nature of the pangenome of CC95 was investigated using WGS data for 200 CC95 strains. These included a collection of strains from Australia ($n = 114$) and France ($n = 17$) for which WGS data (Roche 454 GS FLX+ system, Illumina MiSeq, and Illumina HiSeq2000) were available plus a collection of CC95 strains ($n = 66$) as identified by Deshpande et al. (55) for which WGS data were available from NCBI, as well as three CC95 reference strains (APEC01, S88, and UT189) (Table S1). The assemblies and annotations for all non-NCBI strains are available in Enterobase (<http://enterobase.warwick.ac.uk/>).

The assembled genomes were annotated using Prokka (41), and ROARY (42) was used for the pangenome analysis. The Harvest suite of tools (43) was used to align the strains and visualize the inferred phylogenetic relationships of these 200 CC95 strains. As expected, the phylogeny revealed significant structure within CC95, and the observed clustering largely correlated with a strain's serotype (Fig. 3).

CC95 *in silico* characterization. Where required, the 200 CC95 isolates were assigned to sequence types (STs) using the University of Warwick multilocus sequence type (MLST) scheme (<http://enterobase.warwick.ac.uk/>). The Center for Genomic Epidemiology (CGE) website (<http://www.genomicepidemiology.org>) was used to characterize the strains, using the ResFinder (44) and SeroTypeFinder (45) tools. The presence of mutations in *gyrA* and *parC* associated with fluoroquinolone resistance was determined using RGI (<https://card.mcmaster.ca/analyze/rgi>) (46). The presence of 40 virulence factor genes (Table S1) was determined using a BLAST search (CLC Genomics Workbench v9).

Subgroup detection using PCR-based typing. SCOARY (47) was used to identify genes found to be present in virtually all members of a subgroup and rare or absent in all other subgroups. The resulting gene sets were then examined for genes likely to be suitable for PCR targeting.

A hypothetical protein-coding gene was found to be present in all subgroup A strains and absent in all but three other CC95 strains (B525, E8766, and E9345). A different hypothetical protein-coding gene was found to be present in all subgroup C strains, although this gene was also present in three subgroup D strains. A glycosyltransferase group 2 family protein-coding gene was unique to subgroup B strains. Subgroup D strains were defined by the presence of *yjgJ*, a putative transcriptional regulator-coding gene. Subgroup E strains were defined by the *E. coli* restriction-modification enzyme type I M subunit (*hsdM*) gene. The nucleotide sequences for these genes are presented in Table S2 in the supplemental material.

Primers were designed to specifically target each of these genes, such that fragments of each unique gene could be amplified in a single multiplex PCR (see Table S3 in the supplemental material). Bidet et al. (48) developed a CC95-specific PCR based on the presence of an open reading frame (ORF) of unknown function that they designated *svg*. The *svg* primers were included in the primer pool; *svg* serves as both a positive control for the subgrouping multiplex PCR and an additional verification of an isolate's identity as CC95. All PCRs used a 20- μ l volume containing 4 μ l of 5 \times MyTaq Red buffer (supplied with MyTaq *Taq* polymerase [Bioline]), 2 U of *Taq* polymerase, 1.2 μ l of DNA (at approximately 100 ng/ μ l), and 20 pmol of each primer. The PCR conditions were denaturation for 4 min at 94°C, 30 cycles of 5 s at 94°C and 20 s at 59°C, and a final extension step of 5 min at 72°C.

Distribution of CC95 and CC95 subgroups. A number of existing *E. coli* strain collections acquired for a variety of reasons and over a number of years from hosts living in Australia, France, and the United States were screened to determine which strains in the collections belonged to CC95. Additional details of these collections are presented in the Results section. The phylogroup membership of these strains had been determined previously. CC95 identity was determined by either multilocus sequencing typing or PCR-based screening using one or more of the published CC95 detection methods (7, 48, 49). The methods used for PCR-based virulence factor screening are described in the references cited for each of the strain collections used in this study.

All CC95 strains identified in the various strain collections were then screened using the CC95 subgroup multiplex PCR, together with PCR-based O typing (50) and H typing for H7 (51) and H4 (52). Any strain yielding an *svg* product together with a subgroup C and subgroup D PCR product was considered a subgroup D strain (Fig. 5).

Intrinsic extraintestinal virulence. The virulence of CC95 strains was tested in a mouse model of sepsis following neck subcutaneous inoculation of 2×10^8 bacteria as described by Johnson et al. (24). Briefly, 10 outbred female Swiss mice were inoculated per strain, and death was monitored up to 7 days after inoculation. In each experiment, two *E. coli* control strains were systematically tested: K-12 strain MG1655, which does not kill mice, and strain CFT073, which kills 100% of inoculated mice. Experiments conducted in France (27 strains belonging to all subgroups) followed the European and national regulations for housing and care of laboratory animals after pertinent review and approval by the Bioethics Committee at Santiago de Compostela University and by the French Veterinary Services (certificate no. A 75-18-05). Experiments conducted in Minnesota (31 strains belonging to subgroups A, B, and D) followed federal regulations for ethical care and use of laboratory animals, with approval by the local Institutional Animal Care and Use Committee (protocol 120603).

Statistical analysis. Factors influencing the relative frequency of CC95 subgroups were investigated using either contingency table analysis or nominal logistic regression where appropriate. For analyses involving multiple factors, nonsignificant terms were dropped using backwards elimination, and only significant terms were reported.

Accession number(s). The raw sequence read files have been deposited in NCBI and are associated with BioProject PRJNA385370, accession no. SRX2786117 to SRX2786219.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mSphere.00168-17>.

TABLE S1, XLS file, 0.1 MB.

TABLE S2, DOCX file, 0.1 MB.

TABLE S3, DOCX file, 0.1 MB.

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