Plasmid 83 (2016) 8–11

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Whole genome sequencing of diverse Shiga toxin-producing and non-producing *Escherichia coli* strains reveals a variety of virulence and novel antibiotic resistance plasmids

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ARTICLE INFO

Article history:
Received 25 August 2015
Received in revised form 21 December 2015
Accepted 29 December 2015
Available online 31 December 2015

Keywords:
Genome sequencing
STEC
Plasmids
Antibiotic resistance
Virulence genes

ABSTRACT

The genomes of a diverse set of *Escherichia coli*, including many Shiga toxin-producing strains of various serotypes were determined. A total of 39 plasmids were identified among these strains, and many carried virulence or putative virulence genes of Shiga toxin-producing *E. coli* strains, virulence genes for other pathogenic *E. coli* groups, and some had combinations of these genes. Among the novel plasmids identified were eight that carried resistance genes to aminoglycosides, carbapenems, penicillins, cephalosporins, chloramphenicol, dihydrofolate reductase inhibitors, sulfonamides, tetracyclines and resistance to heavy metals. Two of the plasmids carried six of these resistance genes and two novel IncHI2 plasmids were also identified. The results of this study showed that plasmids carrying diverse resistance and virulence genes of various pathogenic *E. coli* groups can be found in *E. coli* strains and serotypes regardless of the isolate's source and therefore, is consistent with the premise that these mobile elements carrying these traits may be broadly disseminated among *E. coli*.

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1. Introduction

Shiga-toxin producing *Escherichia coli* (STEC) is characterized by the production of Shiga toxins (Stx), a family of related protein toxins encoded by lambdoid prophages with two major types designated Stx1 and Stx2 (Perna et al., 2001; Tarr et al., 2005). Among STEC, O157:H7 is most important serotype that causes food-borne outbreaks worldwide (Mead and Griffin, 1998; Perna et al., 2001), but other STEC serotypes have also caused infections and severe human illnesses (Brooks et al., 2005). STEC strains that cause severe diseases often produce the intimin protein that enable the pathogen to adhere to intestinal epithelial cells (Mead and Griffin, 1998; Tarr et al., 2005), but they also carry a variety of plasmids, some of which encode for putative virulence factors and others, for antibiotic resistance (Johnson and Nolan, 2009). Among the best studied STEC plasmid is the large pO157 plasmid in O157:H7, which is approximately 60 MDa and 92 kb in size (Johnson and Nolan, 2009). Similar large plasmids are present in other STEC serotypes, and some of these, like the plasmid in serotypes O26:H11, O26:H30, O145 and O157:H7 Sakai have been sequenced to determine the presence or absence of specific genes, such as *ehxA* (enterohemolysin), *espP* (serine protease) and *katP* (catalase peroxidase) (Machino et al., 1998; Brunder et al., 1999, Fratamico et al., 2011; Yan et al., 2012). But few have examined the plasmid contents of other *E. coli* strains and serotypes. Since plasmids are highly mobile genetic elements that can be transferred, we examined the overall plasmid content of various STEC and non-STEC *E. coli* serotypes isolated from various sources and sequenced these plasmids to determine the diversity of genes that are found on these plasmids.

2. Materials and methods

The 26 bacterial strains used in this study are from 18 different O-serogroups and comprised of 22 STEC and four non-STEC strains. Of these, 21 strains were isolated from different hosts, including seven from cows, eight from humans, three from pigs, and one each from horse, rabbit, and goat. Four environmental strains were isolated from surface waters in Maryland (Feng et al., 2010) and one strain was isolated from a sample of contaminated food. The metadata on these strains are shown in Table 1. The flagellar (H) type of some strains was identified, but others were not fully serotyped or were non-motile (NM) and so, the H types are not shown (Table 1). All strains were whole genome sequenced using a combination of 8 kb paired-end 454 pyrosequencing

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http://dx.doi.org/10.1016/j.plasmid.2015.12.001
0147-619X/Published by Elsevier Inc.

Table 1
Characteristics and accession numbers of sequenced strains.

BioProject	Accession	Strain	Host	Serotype	No. Plasmids	Plasmid replication types	Virulence plasmid-endoded traits[a]
PRJNA51085	AEZI00000000	1.2741	Cow	O2:H4	2	FIB; Frep	Ehx, STa, TraT
PRJNA51087	AEZJ00000000	97.0246	Cow	O5	4	FIB; 3 not classified	Ehx
PRJNA51089	AEZK00000000	5.0588	Cow	O8	1	FIB	Ehx, Iuc, EspP, TraT
PRJNA51091	AEZL00000000	97.0259	Cow	O11	2	FIA; not classified	Ehx, KatP, TraT
PRJNA51097	AEZO00000000	1.2264	Goat	O76	1	FIB	Ehx, TraT
PRJNA51101	AEZQ00000000	96.0497	Human	O91	1	FIA	Ehx, EspP, Pet, SigA, Sat
PRJNA51103	AEZR00000000	99.0741	Food	O91	1	FIB	Iuc, TraT
PRJNA51105	AEZS00000000	3.2608	Horse	O103:H2	1	FIB	Ehx, TraT
PRJNA51107	AEZT00000000	93.0624	Human	O103:H6	1	FIB	Ehx, TraT
PRJNA51109	AEZU00000000	4.0522	Cow	O111	4	Frep; B/O; 2 not classified	Ehx, KatP, TraT
PRJNA51111	AEZV00000000	JB1-95	Human	O111	4	Frep; 3 not classified	Ehx, KatP, TraT
PRJNA51113	AEZW00000000	96.154	Human	O113	1	FIB	Ehx, TraT
PRJNA51115	AEZX00000000	5.0959	Unknown	O121:H19	0		None
PRJNA51119	AEZZ00000000	9.0111	Human	O128:H2	3	Frep; 2 not classified	Ehx, TraT
PRJNA51121	AFAA00000000	4.0967	Rabbit	O145:H2	4	HI2; Frep; I1; not classified	pAPEC
PRJNA51123	AFAB00000000	2.3916	Pig	O147	1		Ehx, ST, TraT
PRJNA51125	AFAC00000000	3.3884	Cow	O153	2	FIB; not classified	Ehx, EspP, TraT
PRJNA51127	AFAD00000000	2.4168	Water	O157:H16*	0		None
PRJNA51129	AFAE00000000	3.2303	Water	O157:H16*	1	FIB	Ehx,EspP,Pet, SigA, Sat
PRJNA51131	AFAF00000000	3003	Human	O157:H45*	0		
PRJNA51133	AFAG00000000	TW07793	Human	O157:H39*	0		
PRJNA51135	AFAH00000000	B41	Pig	O101:NM	1	FIA	STa, Paa, TraT
PRJNA51137	AFAI00000000	900,105(10e)	Cow	O26:H11	1	not classified	None
PRJNA190228	AEZP00000000	97.0264	Cow	O88:H25	1	FIB	Iuc, TraT
PRJNA190229	AEZY00000000	9.1649	Pig	O2	1	A/C	None
PRJNA66221	SRX072955	C691-71 (14b)	Human	O128:H21	1	HI2	pAPEC

* O157 non-H7 strains — not STEC.
[a] Virulence and putative virulence genes identified by BLAST to Virulence Factor Data Base (VFDB): Ehx: enterohemolysin (*ehxCABD*) operon, Esp: serine protease (*espP*), KatP: catalase peroxidase (*katP*); Iuc: aerobactin synthesis (*iuc*) operon; TraT: complement resistance; Pet: plasmid encoded enterotoxin of enteroaggregative *E. coli* (EAEC); pAPEC: plasmid of avian pathogenic *E. coli* (APEC) which contains the virulence factors of APEC; Sat.: secreted autotransporter toxin; Paa: porcine attaching and effacing associated factor; STa: heat stable enterotoxin; SigA: secretory immunoglobulin A.

and sequencing by synthesis with Illumina (http://gsc.jcvi.org). The fragment libraries obtained were assembled with the Celera Assembler software (Miller et al., 2008). Genomes were improved to high quality draft status using JCVI's automated closure tools. Most of the larger plasmids were not assembled into a single contig, however scaffolding was possible by manual curation using paired-read status and read-depth. The physical mate-pair link and the difference in read depth was sufficient to distinguish among plasmids where multiple plasmids existed. Replicon typing was done by in silico PCR (CLC Genomics Workbench) using primers described by Johnson et al., 2007.

3. Results and discussion

The presence of plasmids in the strains were identified by contigs that showed typical plasmid characteristics such as (i) evidence of circularity as determined by mate-pair read status, (ii) annotations commonly associated with plasmids, and (iii) sequence similarity with other *E. coli* plasmids. We identified 39 new plasmids whose sequences have not been published previously. These ranged from 5–250 kb in length and were assembled into 1–50 contigs [Supplementary File 1]. The number of plasmids present ranged from 0 to 4 per strain, with 14 strains carrying only 1 plasmid, and 4 strains with 4 plasmids. These plasmids were designated by using the prefix "p" followed by the strain name and in the case of strains with multiple plasmids, they were numbered from the largest to the smallest [e.g. pEc4.0967–1, pEc4.0967–2, etc.]. There were four strains that did not carry any plasmids; one was an O121:H19 strain and the other three were O157 non-H7 strains that were not STEC. These included strain 2.4168, an O157:H16 strain isolated from surface waters and appeared to be wild type *E. coli*; strain 3003, a human isolate of serotype O157:H45 that had both the *eae* and *bfp*A genes that encode for intimin and bundle forming pilus, respectively, and therefore, is an enteropathogenic *E. coli* (EPEC), and strain TW7793 from Argentina, a human isolate of serotype O157:H39 that only had the *eae* gene and so, is an atypical EPEC strain (Feng et al., 2010). As a whole, the 39 plasmids identified from all the strains grouped into 11 known replication classes, of which 21 were of the F-replicon type [Frep, FIB, FIA, or FIIA]. There were seven plasmids, which could not be assigned to any known class [Table 1]. These results are consistent with other large-scale plasmid typing efforts that were done in *Enterobacteriaceae* (Carattoli et al., 2005) and in *E. coli* (Marcade et al., 2009), where the majority of the plasmids identified were of F-replicon type. The genes involved in plasmid transfer and function were highly conserved within each class of plasmids, showing >80% sequence identity. The many virulence and putative virulence genes, including enterohemolysin, proteases, lipid A modification enzymes, as well as for antibiotic resistance were identified by BLAST using the Virulence Factor Database (Chen et al., 2012). These genes were present in many of the plasmids irrespective of their replicon type, or the sources from which the strains were isolated [Table 1]. Where possible, after in silico reconstruction of plasmids based on mate-pair read scaffolding, the genes or operons within plasmids were generally organized similar to known sequenced plasmids.

The large STEC plasmid (>70 kb) that carried the enterohemolysin operon (*ehxCABD*) was common among the strains tested and found in 15 of the 26 strains, including in one non-STEC strain. Of these, four also had the secreted serine protease gene (*espP*), and three had the catalase-peroxidase gene (*katP*), but none had both *espP* and *katP* [Table 1]. A study showed these large STEC plasmids to be very heterogeneous, and their gene content can vary in different strains (Brunder et al., 1999).

The *traT* gene encodes for a major outer membrane protein (OMP) that enables complement and serum resistance and is thought to contribute to bacterial survival under adverse conditions, including mammalian innate immunity (Waters and Crosa, 1991). The *traT* gene can be found in many enteric bacteria, including *E. coli*, *Salmonella*, *Shigella*, and *Klebsiella* (Montenegro et al., 1985). In our study, the *traT* gene was found in 15 of the *E. coli* strains examined, and therefore was also very common. The *traT* gene is located in the F transfer region (Frost et al., 1994) and as expected, most of the plasmids we found that carried *traT* were of F replicon type. In some STEC serotypes like

Table 2
Plasmid distribution and resistance profiles.

Plasmid	Antibiotic resistance[a]	Heavy metal resistance[b]
pEcB41	*sul2*	Hg
pEc40967-4	*tetb*	Ar/Te
pEc970246	*teta*	Fe/Mg
pEc990741	*ant3ia*; *aph3ia*; *sul3*; *teta*	Fe/Mg
pEc970264	*aph33ib*; *aph33ia*; *aph6id*; *sul2*; *tetb*	Fe
pEc40522-4	*teta*; *dfra12*; *aph3ia*; *sul1*; *macb*	Hg
pEc23916	*ant3ai*; *aph3ia*; *cml_e1*; *sul3*; *teta*	Cu/Hg
pEc91649	*aph33ib*; *aph6id*; *bl1_cmy2*;*cml_e3*; *sul1*; *sul2*; *teta*	Hg

[a] Gene denominations according to ARDB (10): *teta* and *tetb*: tetracycline; *aph33ia*: Streptomycin; *ant3ia*: Spectinomycin; *aph33ib*: Kanamycin; *sul1* and *sul2*: Sulfonamides; *macb*: Macrolides; *cml_e1* and *cml_e3*: Chloramphenicol; dhfr: Trimethoprim; bl1_cmy2: Carbenicillin.
[b] Fe: Iron, Ni: Nickel; Hg: Mercury; As: Arsenic; Te: Tellurite; Cu: Copper.

O26:H11 and O26:H30, the *traT* gene is on the large STEC plasmid that also carry *ehxA* (Fratamico et al., 2011). Our results suggest that this may be the case with other *E. coli* serotypes as well, since 12/15 strains that had *traT* also had *ehxA*.

Several strains were found to carry virulence genes from various pathogenic *E. coli* groups. For example, a non-STEC strain carried the *ehxA* gene that is most common in STEC, and three STEC strains had both the *ehxA* gene and the stable toxin (ST) gene of enterotoxigenic *E. coli* (ETEC). We also found another STEC strain that carried both the *ehxA* and the plasmid-encoded toxin (Pet) gene of enteroaggregative *E. coli* (EAEC) (Yamazaki et al., 2000). Although the *ehxA* gene is commonly associated with STEC, it can be found in wild type *E. coli* isolated from surface waters (Boczek et al., 2005), in atypical enteropathogenic *E. coli* strains (Cookson et al., 2007), as well as in *E. coli* strains isolated from fresh produce, which carried both *ST* and *ehxA* genes (Feng and Reddy, 2014). So our results are consistent with those of other studies that *ehxA* is present in various groups of *E. coli*. Similarly, the porcine attaching and effacing (Paa) gene that was originally identified in attaching and effacing *E. coli* (AEEC) was found in a strain that also had the *STa* gene of ETEC [Table 1], in agreement with the report by Leclerc et al., 2007. Lastly, two of the STEC strains in our study were found to carry the large pAPEC plasmid that is common in avian pathogenic *E. coli* (APEC) strains (Mellata et al., 2009). The identification of these various *E. coli* strains that are carrying virulence traits or combinations of virulence traits of different pathogenic *E. coli* groups are consistent with the premise that plasmids are highly mobile and can be transferred, which not only contributes to genomic diversity of strains but also potentially expand the pathogenic capacity of recipient strains (Johnson and Nolan, 2009).

Among 8 of the newly-sequenced plasmids, there were a total of 29 genes that mediated resistance to antibiotics as annotated in the Antibiotic Resistance Database (Liu and Pop, 2009) [Table 2]. These resistance genes belonged to several major classes of antibiotics including: carbapenems, aminoglycosides, penicillins, cephalosporins, chloramphenicol, dihydrofolate reductase inhibitors, sulfonamides and tetracyclines. Most plasmids carried more than one resistance gene and thus presumably, conferred resistance to multiple classes of antibiotics. The most common type of resistance genes were those encoding for aminoglycoside modification enzymes [18 genes total], including adenylation, phosphorylation, and acetylation, followed by efflux pump transporters [10 genes total], including several used for detoxification of heavy metals, like mercury, zinc, copper, tellurite, and arsenic/arsenate [Table 2]. Though antibiotic resistance genes were commonly found adjacent to each other, no integron sequences were detected in any of these newly identified plasmids. Three of the plasmids that carried multiple drug resistance genes also carried genes involved in repair of UV damage.

Two of the plasmids were identified to be of the H incompatability group (IncHI2), which are known to play a role in the acquisition of antibiotic resistance and are also frequently associated with virulence in Proteobacteria (Johnson et al., 2006). The 2 IncHI2 plasmids were identified in STEC strains Ec4.0967, a rabbit isolate of O145:H2 serotype and C691-71(14b), a human isolate of O128:H21 serotype. Johnson et al., 2006 reported that extraintestinal APEC can carry the pAPEC plasmids that are IncHI2 type and encode for resistance to as many as eight antimicrobial agents. Hence, we compared the sequences of our two plasmids to that of pAPEC-O1-R, an IncHI2 plasmid of 241,387 bp in size that was found in an APEC strain (Johnson et al., 2006). The pEc40967-1 was smaller (215,572 bp) with an overall GC content of 44.7%, which is less than that of the chromosome [49.5%] or that of pAPEC-O1-R [46%] (Johnson et al., 2006). Previous analysis of IncHI2 plasmids had suggested that antibiotic and heavy metal resistance can be acquired horizontally by these plasmids in dedicated hotspots, while the rest of the plasmid body is highly conserved (Johnson et al., 2006; Garcia-Fernandez and Carattoli, 2010). Consistent with those reports, there were large areas of conservation and synteny between pAPEC-O1-R and pEc40967-1 that roughly covered 82% and 93% of each plasmid, respectively, and included all of the previously identified constant regions found in IncHI2 plasmids (Garcia-Fernandez and Carattoli, 2010). The two regions that were not conserved between the plasmids were the region in pAPEC-O1-R that encodes for streptomycin resistance, which was absent in pEc40967-1 and conversely, the region encoding for arsenic resistance in pEc40967-1, which was absent in pAPEC-O1-R [Fig. 1]. The plasmid pEcC691-71 was assembled into 7 contigs (202,180 bp) and also shared a high degree of synteny with pAPEC-O1-R and pEc40967-1. But, pEcC691-71 lacked the arsenic resistance operon found in pEc40967-1 and also lacked the antibiotic resistance genes and the silver and copper resistance operons that were located in the same region in pAPEC-O1-R. Since all these resistance genes were localized within the same region on these plasmids, this region may be a hotspot for the insertion of resistance genes (Johnson

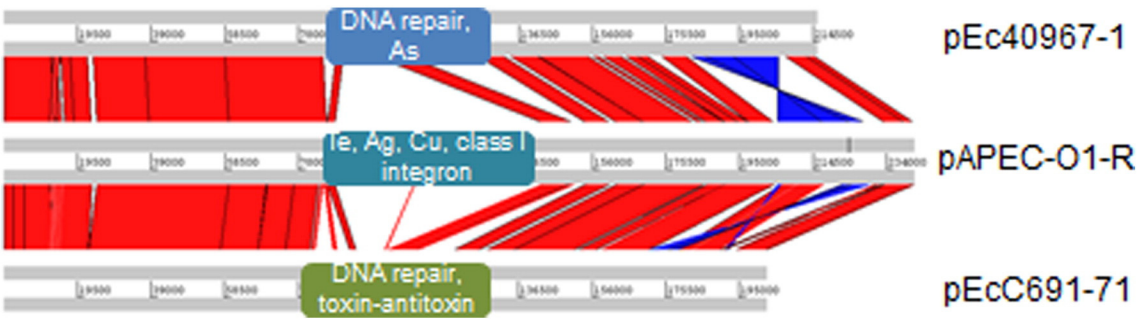

Fig. 1. Nucleotide alignment of IncHI2 plasmids pEc40967-1, pAPEC-O1-R and pEcC691-71. Alignments were performed in Artemis Comparative Tool (Abbott et al., 2005) and the numbers on the strands represent nucleotide coordinates. Synteny and identity along plasmid backbones are shown with red and blue solid lines. The red lines signify alignment of positive strands and blue lines signify inversions. Areas absent of lines indicate 'hotspots', where different resistance genes are carried by different strains. The functions and resistance profiles labeled are: As, arsenic/arsenate resistance, Te, tellurite; Ag, silver efflux; Cu, copper efflux; class I integron encodes resistance to aminoglycosides and sulfonamide (Johnson et al., 2006).

et al., 2006). The two IncHI2 plasmids we identified showed a lot of sequence similarities but also some differences, hence they were further characterized with the in silico double-locus sequence typing (DLST) method developed for IncHI2 plasmids (Garcia-Fernandez and Carattoli, 2010). Analysis of the two loci in the conserved region used for typing showed that pEc40967-1 was very similar to plasmid R478 that was isolated from *Serratia marcescens* (Gilmour et al., 2004) and therefore, these belonged in the same pDSLT type of IncHI2 plasmids. In contrast, the two same loci in pEcC691-71 were distinct from the others and therefore, pEcC691-71 may represent a new pDLST type of IncHI2 plasmid.

This study showed that genes encoding for resistance to antibiotics, heavy metal, UV light, and complement are commonly found on *E. coli* plasmids. The presence of these genes however, is not indicative that they are actually expressed. For example, the *ehxA* gene on the large plasmid of sorbitol-fermenting O157 STEC strains is not expressed and so enterohemolysin is not produced (Karch and Bielazewska, 2001). Similarly, *E. coli* strains can have plasmids with an intact promotor and genes that encode for resistance to streptomycin, ß-lactamase, sulfonamides and tetracycline, but not all expressed these genes, presumably due to a chromosomal transcriptional control that silenced the expression of plasmid genes (Enne et al., 2006). However, the genetic evidence that a large diversity of virulence and resistance genes carried by plasmids of the different *E. coli* serotypes is consistent with the premise that plasmid transfer, probably catalyzed by mobile elements, including transposases, is common among *E. coli* and that these strains, regardless of sources of isolation, may carry plasmids with multiple resistance genes, as well as trait genetic markers of various pathogenic *E. coli* groups.

Nucleotide sequence accession numbers

The information on all plasmids used in this analysis and the *E. coli* Whole Genome Shotgun project and GenBank accession numbers are described in Table 1. The entire sequence of all plasmids is available at ftp://ftp.jcvi.org/STEC/plasmids/.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plasmid.2015.12.001.

Acknowledgments

This project has been funded in whole or in part with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services under contract number HHSN272200900007C. The authors also thank Mark Adams, Derrick Fouts and William Nierman for their review of this manuscript.

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