Genetic characterization of extended-β-lactamase (ESBL) plasmids captured from dairy manures

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ABSTRACT

This study was to assess the gene diversity and characterize a large set of plasmids harboring extended β-lactamase (ESBL) genes from raw and digested dairy manure. A total of eighty-four plasmids that were captured in this *E. coli* recipient were sequenced using Illumina MiSeq sequencing technology. Twenty-four plasmids of interest were subsequently sequenced using MinION technology in order that a hybrid assembly could be performed on short- and long-read sequences to circularize and complete these plasmids. The size of sequenced plasmids ranged between 40 and 260 kb with various incompatibility groups: IncC, IncI1, IncN, IncY, IncB/O/K/Z, IncX1, IncHI2, IncHI2A, IncFIB(K), IncFII. A variety of extended β-lactamase genes were identified: $bla_{\text{CTXM -1}}$, $bla_{\text{CTXM -14}}$, $bla_{\text{CTXM -15}}$, $bla_{\text{CTXM-27}}$, $bla_{\text{CTXM-55}}$, $bla_{\text{CTXM-61}}$, $bla_{\text{PER-1,}}$ $bla_{\text{IMP-27}}$. Interestingly, the $bla_{\text{IMP-27}}$ gene, a novel metallo-β-lactamase discovered in the last decade, was found located on an integrated region in the host chromosome. And one plasmid carrying the $bla_{\text{CMY-2}}$ gene, an AmpC gene, also expressed ESBL phenotype. Four virulence factors, including *cia, cib, traT* and *terC,* were detected on some of these plasmids. In addition, six type-2 toxin-antitoxin systems were detected: MazF/E, PemK/I, HipA/B, YdcE/D, RelB/E and HigB/A. Twenty-two out of twenty-four complete plasmids carried putative prophage regions; and most of prophage hits were marked as incomplete, except that the largest plasmid pT525A and the IncY plasmid pT415A had prophage hits with higher scores.

IMPORTANCE

The widespread of antibiotic resistant bacteria is largely due to the exchange of mobile genetic elements such as plasmids. Plasmids harboring extended β-lactamase (ESBL) genes originated from dairy manure potentially become entrained in manured soil, which subsequently enter the human food chain. Currently there is a lack of detailed information on these plasmids in the

environment, specifically in dairy manure. This study unveils the abundance and diversity of ESBL-carrying plasmids from both raw and digested manures which were captured in *gfp*-labelled *E. coli* CV601. In addition, the study provides insightful information of plasmid characteristics including incompatibility groups, ESBL genes combined with other resistance genes, mobile genetic elements (transposons, insertion sequence), toxin-antitoxin systems, virulence factors and prophage sequences.

INTRODUCTION

Extended-β-lactamase (ESBL) genes have been a matter of undoubtedly grave public-health concern due to their ability to hydrolyze third-generation cephalosporins (e.g. cefotaxime, ceftriaxone, ceftazidime, or cefepime) and monobactams (aztreonam) (1). A dramatic increase in the number of multidrug-resistant Enterobacteriaceae (mostly *Escherichia coli*) that produce extended-spectrum β-lactamases (ESBLs), such as the CTX-M enzymes, has been reported since the 1990s (2). ESBL genes has been widely disseminated via mobile genetic elements such as plasmids, insertion sequences, transposons. Plasmids carrying ESBL genes are ubiquitous in environments including manure, manured soil, wastewater treatment plants and aquaculture (3-8).

Bacterial toxin-antitoxin (TA) systems are pairs of genes encoding a toxin protein and its corresponding antitoxin protein which can be found on either chromosomes or plasmids in free-living bacteria (9, 10). The first TA operon was found on plasmid R1 about three decades ago, and was shown to play an important role in plasmid stability by the post-segregational removal of plasmid-free cells (11, 12). The ccd system on the F plasmid, the most widely studied system, was even employed in DNA cloning strategies (13). Depending on the molecular structures and mechanisms of action, three types of TA operon were presented: Type I, II, and III (12). The type II TA system, also termed as the addiction system, consisted of at least ten current families such as MazE-MazF, RelE-RelB, YefM-YoeB, and MqsR-MqsA (12, 14). Despite their ubiquity in bacteria, TA systems on manure-originated plasmids are not well-understood.

Virulence factors mainly accounts for bacterial pathogenicity which causes diseases in hosts such as plant, animals and human (15, 16). They can be found on either pathogenicity islands in the genome of pathogenic bacteria or on plasmids (17, 18). Virulence-associated plasmids in *E. coli*

were associated with six pathotypes enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), extraintestinal pathogenic *E. coli* (ExPEC) (17). Nine out of 26 plasmid incompatibility groups which mostly fall into group F were known to carry virulence genes, and there is no doubt this number would keep rising as novel plasmid groups continue to be identified (17, 19).

Prophages are bacteriophage sequences that normally integrate into bacterial chromosome and largely contribute to bacterial adaptation and evolution by enabling the horizontal genetic exchange (20, 21). A few prophages (e.g. P1, N15, LE1, □20, and □BB-1), however, are able to independently replicate in the lysogen as low-copy-number plasmids (22). Prophages have an average size between 29 to 78 kb, which probably constitute about 0.6 to 1.8 % of the host chromosome (23). Therefore, plasmids of megasize (> 100kb) can easily capture prophage regions via either homologous recombination or the movement of insertion sequences/ transposons. Recent evidences suggested that the plasmid pMCR-1-P3, an IncY plasmid, was the outcome of homologous recombination event between a plasmid and a prophage region located in the *E. coli* genome (24).

The aim of the study was to extensively and intensively analyze genetic characteristics of eighty-three sequenced ESBL plasmids originated from dairy manure including a subset of twenty-five plasmids reported previously (25). The study also revised the comparison of plasmids from raw with those from digested manure in a larger set of data. Overall this study provide insightful information of plasmid characteristics such as plasmid size, virulence factor, TA systems, incompatibility groups, mobile genetic elements and antibiotic resistance genes

RESULTS

Description of sequenced plasmids harboring ESBL genes from raw and digested manures

In this study, a total of 83 plasmids harboring ESBL genes were grouped based on their incompatibility groups, ESBL genes and other resistance genes (Table 1). A detailed information of 25 plasmids was reported previously (manure paper); while the rest of them can be found in supplementary material of this study (Table S1). Twenty distinct plasmid profiles carrying eight ESBL genes in combination with other resistance genes were identified (Table 1). Eleven transconjugants' whole genomes were further sequenced on the MinION long-read sequencing platform so that hybrid assembly could be used to completely close the plasmids carried by these transconjugants. Maps of these complete plasmids were presented in Fig. 1 & Fig. S1.

Among 83 sequenced plasmids, 35 of them were from raw manure, and 48 from digested manure (Table 1). The most frequent plasmid (pT545A) carried *bla*$_{\text{CTXM-15}}$ gene along with other nine resistance genes: *aac(6')-Ib-cr, aph(3'')-Ib, aph(6)-Id, bla*$_{\text{OXA-1}}$, *catB4, dfrA1, floR, lnu(G), sul2*. This plasmid was found in both raw and digested manures from all participating farms. The second most frequent plasmid carrying *bla*$_{\text{CTXM-1}}$ gene (pT115A) was found in both raw and digested manure from four out of six participating farms.

Other 18 less frequent plasmids were found in either raw (four plasmids) or digested manures (fourteen plasmids) (Table 1). Four plasmids from raw manure individually carried following ESBL genes: *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-55}}$, *bla*$_{\text{PER-1}}$. Meanwhile, thirteen out of fourteen plasmids from digested manure individually carried following ESBL genes: *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-14b}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-27}}$, *bla*$_{\text{CTX-M-55}}$. Interestingly, one conjugative plasmid (pT598A) from digested manure did not carry any ESBL genes. However, the *bla*$_{\text{IMP-27}}$ gene was detected on the host chromosome along with other resistance genes: *aph(6)-Id, strA, sul2, tet(A)* in the transconjugant carrying this plasmid pT598A.

Sizes of hybrid assembled plasmids ranged between 40 and 260 kbs; the most frequent plasmid (pT545A) had the size of 100 kbs. Incompatibility groups identified in this study were IncC, IncI1, IncN, IncY, IncB/O/K/Z, IncX1, IncHI2, IncHI2A, IncFIB(K), IncFII(pHN7A8). A variety of mobile genetic elements that were located in areas surrounding resistance genes were identified including Tn*3*, Tn*7*, Tn*As1*, *tnpA*, *IntI1*, *insA*, *insB*, IS*Ec63*, IS*Ec9*, IS*Ecp1*, IS*903B*, IS*3*, IS*5*, IS*26*, IS*91*, IS*110*, IS*5075*, and IS*Vsa3*. Mobile genetic elements that were not located proximal to resistance genes were also identified: IS*3*, IS*5*, IS*66*, IS*21*, IS*911*, IS*Kpn18*, IS*Ec23*.

Description of plasmids with special features

The plasmid pT100A was the only conjugative plasmid carrying an AmpC-type gene (*bla*$_{\text{CMY-2}}$); however, the transconjugant carrying this plasmid expressed ESBL phenotype (Fig 1. & Table S1). The plasmid with a size of 98 kbs had IncI1 incompatibility group. The plasmid did not carry any other resistance genes, but it carried two conjugal transfer genes (*traC*, *traI*). Unlike most of captured plasmids, mobile genetic elements on this plasmid were located far apart from the resistance gene *bla*$_{\text{CMY-2}}$.

The plasmid pT413A with a size of 187 kbs carrying the *bla*$_{\text{PER-1}}$ gene, an ESBL gene, also carried mercury resistance operon (Fig. 2). The mercury resistance operon with a size of approximately 3 kbs consisted of *merA*, *merP*, *merT* and *merR* genes. This operon located in the 79 kb region along with other resistance genes and other mobile genetic elements (integrons, transposon and insertion sequences). The adjacent mobile genetic elements surrounded this operon were transposon Tn7 transposition proteins (TnsB, TnsC).

The conjugative plasmid pT428Al carrying the *bla*$_{\text{CTX-M-14}}$ gene accompanied with the mobilizable plasmid pT428As which did not carry any resistance genes. The pT428Al had a size of 92 kbs, and its accompanying plasmid pT428As had a size of 4kbs. This is the only case

where two plasmids were confirmed to co-transfer into *E. coli* recipient CV601 by hybrid assembly. Using the annotation tool PROKKA, only two genes, *mobA* encoding mobilization protein A and *repE* encoding replication initiation protein, were detected on the smaller plasmid pT428As.

The plasmid pT598A was the only conjugative plasmid did not carry any resistance genes. However, this plasmid carried many conjugal transfer genes: *traB, traC, traD, traG, traI, traK, traJ, traL, traM.* The plasmid pT598A with a size of 42 kbs had no incompatibility group identified. Hybrid assembly of transconjugant's whole genome sequence carrying this plasmid showed a 865kb region integrated into the host chromosome (Fig. 3). This region carried multiple resistance genes (*aph(6)-Id, bla*$_{\text{IMP-27}}$, *strA, sul2, tet(A)*) along with other mobile genetic elements (Tn*As1*, IS*Vsa3*, IS*3*, IS*26*, *intA*). The integrated region was between two chromosomal genes: *aspC* gene encoding aspartate aminotransferase and *asnS* gene encoding asparagine--tRNA ligase.

DNA variations among genotypically similar plasmids

In this study, not all plasmids were sequenced on both short-read and long-read sequencing platforms. Plasmids that were sequenced on both platforms were successfully completely closed via hybrid assembly, hence they could be used as a reference input. Plasmids having similar characteristics (sizes, resistance genes, incompatibility groups) with closed plasmids were further analyzed using Snippy tool to search for SNPs or any DNA variations.

There were very few DNA variation detected among plasmids harboring the *bla*$_{\text{CTX-M-1}}$/ *bla*$_{\text{CTX-M-15}}$/ *bla*$_{\text{CTX-M-27}}$ gene (Table S2, S3 and S4). However, three largest IncHI2-IncHI2A plasmids (>200 kb) harboring the *bla*$_{\text{CTX-M-55}}$ gene were quite distinct from one another (Table S5). Unexpectedly, plasmid pT525A was even more different from plasmid pT594A considering they

both were originated from raw manure of the same farm, and they had resistance genes and incompatibility groups in common.

Toxin-antitoxin systems detected on completely closed plasmids

Six type-2 toxin-antitoxin systems were detected: MazF/E, PemK/I, HipA/B, YdcE/D, RelE/B and HigB/A (Table 2). The MazF-MazE system were the most frequent toxin-antitoxin system detected in our study. It was detected on following plasmids: pT82A, pT101A, pT159A, pT209A, pT267A, pT270A, pT390A, pT545A. The PemK-PemI system was found located on three plasmids: pT156A, pT224A and pT257A. These three plasmids shared a majority of their sequence in common (25). The YcdE-YcdD system were detected on two distinctly different plasmids pT100A and pT593A. The remaining three systems found on less frequent plasmids: HipA-HipB system on pT525A, RelE-RelB system on pT295A and HipA-HipB on pT525A. There was no toxin-antitoxin systems detected on following plasmids: pT199A, pT247A, pT115A, pT428Al, pT428As, pT455A, pT526A, pT570A.

Detection of virulence factor genes on completely closed plasmids

There were four virulence factors detected: *cia, cib, traT, terC* (Table 3). The *cia* gene encoding colicin ia was found on two distinct plasmids pT247A and pT428Al, while the *cib* gene encoding colicin ib was detected on one plasmid pT100A. The *traT* gene encoding complement resistance protein was found on four plasmids pT156A, pT224A, pT257A and pT593A. Among them, pT593A was more distinctly different than the other three plasmids (pT156A, pT224A, pT257A) whose sequences shared a lot in common as shown previously (25). The *terC* gene encoding tellurium ion resistance protein was detected on one plasmid pT525A. There was no virulence factor detected on following plasmids: pT82A, pT100A, pT101A, pT115A, pT159A,

pT209A, pT267A, pT270A, pT295A, pT390A, pT413A, pT428As, pT545A, pT526A, pT570A, pT593A, pT598A, There was no shiga-toxin genes was detected on any of plasmid input.

Detection of prophage sequences on plasmids

A majority of plasmids (22/26) got hits for prophage detection (Table 4): three plasmids got three prophage hits (pT82A, pT390A, pT545A); seven got two prophage hits (pT100A, pT101A, pT247A, pT270A, pT428Al, pT525A, pT593A); 13 got one prophage hit (pT115A, pT156A, pT159A, pT199A, pT209A, pT224A, pT257A, pT267A, pT295A, pT415A, pT455A, pT526A, pT570A). Three plasmids did not have any prophage sequences detected: pT428As, pT598A, pT413A.

There were seven most common phage detected on these input plasmids. PHAGE_Escher_RCS47_NC_042128 was detected in most of them (16/25) while others were less common on our plasmids. PHAGE_Mycoba_Shipwreck_NC_031261 was found on five plasmids. PHAGE_Entero_N15_NC_001901 and PHAGE_Salmon_SJ46_NC_031129 were found on four plasmids. PHAGE_Klebsi_phiKO2_NC_005857 and PHAGE_Cronob_ENT39118_NC_019934 were detected on three plasmids. PHAGE_Acinet_vB_AbaM_ME3_NC_041884 was only detected on the plasmid pT525A.

All input plasmids but two got prophage hits classified as incomplete with a score ≤ 70. The PHASTER tool has its own criteria for scoring prophage regions and classifying them based on their scores: intact (score > 90), questionable (score 70-90), incomplete (score < 70). Two plasmids got hits with scores in a range of 70-90 (questionable), higher scores compared to other plasmids (Fig. 4). These two plasmids included the largest plasmid pT525A with two hits and the

205 IncY plasmid pT415A with one hit. None of the hits had the scores within the range of the intact

206 group (>90).

DISCUSSION

The most prevalent plasmid found among 83 sequenced plasmids was the 104 kb plasmid carrying the *bla*$_{\text{CTXM-15}}$ gene. This result was consistent with our previous observation using restriction enzyme profiles combined with a subset of 25 sequenced plasmids (25). This plasmid was abundantly present in both raw and digested manures across all participating farms; however, the current bioinformatics tool was unable to identify its incompatibility group. This left us wondering if the plasmid adopted some novel incompatibility group which has not been recognized in the database. In a recently published study, the dominant ESBL plasmid isolated from 53 dairy farms located in southwest England was 220-kb IncHI2 plasmid carrying *bla*$_{\text{CTX-M-32}}$ (26). This is interesting because only three out of 83 sequenced plasmids in our study belonged to IncHI2 group and had plasmid sizes larger than 200 kb; nevertheless, they carried *bla*$_{\text{CTX-M-55}}$ gene along with other completely different resistance genes. In addition, SNP analysis on these three plasmids revealed a number of nucleotide modifications including deletion, insertion and

Sequencing another set of plasmids, the number of which was as twice as those in our previous study, allowed us to identify more plasmids that were less frequent (25). The second frequent plasmid, which was found in four out of six farms, especially quite dominant in farm 7, was the 43 kb plasmid of group IncN carrying the *bla*$_{\text{CTX-M-1}}$ gene - the only resistance gene on this plasmid. In addition, plasmids from digested manure were more diverse than those from raw manure. Previously we showed that anaerobic digestion significantly reduced the conjugation frequency of ESBL-carrying plasmids in raw manure, but did not necessarily changed plasmid enzyme restriction profiles (25). The fact that a greater diversity of plasmids obtained from digested manure observed in this study can be explained as following. Firstly, it could be that the most frequent plasmid was overwhelming in raw manure, hence there was less chance for other

plasmids to be selected for further analysis. Secondly, restriction enzyme profile combined with a small sequencing set did not adequately identify other less frequent plasmids. With a larger sequencing set, more distinct plasmids have been revealed in this study.

The *bla*$_{CMY-2}$ gene is considered as an AmpC-type β-lactamase gene, which has been wide-spread around the world (27-29). This gene was mostly found on IncA/C plasmids in *E. coli* and *Salmonella* strains (27, 30, 31). Normally this gene confers AmpC resistance phenotype which hydrolyzes cephamycins as well as other third-generation cephalosporins and does not get inhibited by ESBL inhibitors (i.e. clavulanic acid, sulbactam, tazobactam) (32). Previous studies showed that co-location of AmpC and ESBL genes resulted in a combined ESBL/AmpC phenotype (29, 33, 34). Detection of ESBL phenotype in a strain producing both AmpC and ESBL enzymes could be problematic because clavulanic acid, an ESBL inhibitor used in ESBL confirmatory tests, induces the high level expression of AmpC which, in turn, masks the synergy effects on ESBL (28, 33). However, in our study the *bla*$_{CMY-2}$ gene found on IncI1 plasmid conferred ESBL phenotype in *E. coli* strain. Although ESBL-producing *E. coli* carrying only the *bla*$_{CMY-2}$ gene was reported previously, there was not a clear explanation for this phenomenon (3). IncI1 plasmids carrying the *bla*$_{CMY-2}$ gene were shown to widely spread among *E. coli* (35), and they shared a high degree of sequence similarity when isolated from Enterobacteriaceae with different epidemiological links (36). Unlike other plasmids carrying ESBL genes in this study, insertion sequences were not located proximal to the *bla*$_{CMY-2}$ gene.

The *bla*$_{IMP-27}$ gene, a novel metallo-β-lactamase, was firstly isolated in *Proteus mirabilis* in Ontario, Canada and presented in a conference in 2012 (37). Not until four years later, it was reported in published studies, including those found in unrelated *Proteus mirabilis* clinical isolates from two geographically distinct locations in the United States (38, 39). Another study

253 showed that the gene was located on conjugative plasmids that was transferable from either
254 *Proteus mirabilis* or *Providencia rettgeri* to *E. coli* (40). Not only was this gene found in clinical
255 settings, but it was also recovered from the environment of a swine farrow-to-finish operation in
256 the United States (41). The resistance phenotype to carbapenems and β-lactams conferred by this
257 gene was quite distinct and might vary among host strains (38, 40). In our study, the $bla_{\text{IMP-27}}$
258 gene, along with other resistance genes, was surprisingly found integrated into the host
259 chromosome; while the conjugative plasmid isolated from this *E. coli* host was antibiotic
260 resistance-free. We postulated that the integrated region originally got a ride on the plasmid, and
261 it was then transferred to the host chromosome via homologous recombination as soon as the
262 plasmid entered the host cell. To our best knowledge, this is the first time this gene was found in
263 environmental samples in Canada.

264 TA systems, which were first detected on plasmids and later in bacterial chromosomes,
265 play a vital role in plasmid stability as well as other positive roles in bacterial physiology,
266 pathogenicity, and evolution (12, 42, 43). All TA systems detected in this study belonged to
267 Type II TA systems which has been most extensively studied (12). TA systems were supposedly
268 associated with stress-induced environment conditions which enabled stress-responsive proteases
269 to degrade the antitoxin protein in Type II TA systems and free the toxin protein from the TA
270 complex, resulting in cell growth inhibition or cell death (12, 14). The most frequent toxin-
271 antitoxin system found in our study was the MazF-MazE system because this system was located
272 on the most prevalent plasmid. Two TA systems detected in the study, MazF-MazE and RelE-
273 RelB, belonged to super-families that were shown to be abundant and present on plasmids (42-
274 44). Several TA systems, such as HipB-HipA or RelE-RelB, also caused the generation of

persister cells, which went to a dormant state, in the presence of antibiotics, thus survived and became immune to antibiotics (9, 45-48).

Virulence factors detected on plasmids in this study were mostly related to colicin-producing genes (*cia*, *cib*) and the *traT* gene encoding outer membrane complement resistance protein. Colicins Ia and Ib are very similar structurally and able to absorb to common receptor sites on the bacterial outer membranes (49-51). Yet they do not share immunity specificity, hence cells are immune to either colicin Ia or Ib depending on which colicin gene they carry (49). Colicins inhibit protein and nucleic acid biosynthesis and uncouple electron transport from active transport, resulting in the loss of cellular potassium and magnesium which causes cell death (49). The virulence-associated non-conjugative plasmid carrying a *traT*-like gene was first identified in *Salmonella typhirium*; however, the *traT* gene was also found located within the transfer operon of conjugative F-like plasmids in *E. coli* (52, 53). The *traT* gene, one of two F cistrons, prevents the formation of cell contacts, and thus inhibits DNA transfer within the cell population (52). This gene is also needed for the resistance to serum bactericidal activity in *S. typhirium* and *E. coli* (53, 54). The *cia*, *cib* and *traT* genes were found on less frequent plasmids in this study, suggesting that these plasmids were limitedly accessible and only transferable among hosts of particular genetic backgrounds.

Polluted environments such as manured soil, animal farming, waste water and aquaculture can serve as a hot spot for co-selection of metal and antibiotic resistance (55-57). Evidences for metal-driven co-selection of multiple antibiotic resistance via co-resistance and cross-resistance mechanisms were well-documented (55, 56). Co-resistance mechanism occurred when metal/metalloid resistance genes were co-located with antibiotic resistance genes on the same plasmid (55). In this study, gene determinants for resistance to mercury (metal) and

298 tellurium (metalloid), the *merAPTR* operon and the *terC* gene, were detected on two distinct
299 conjugative multidrug resistant plasmids. The genetic linkage of mercury- and antibiotic-
300 resistance genes on conjugative plasmids was demonstrated through mating between
301 *Enterobacteriaceae* family bacteria and doubly genetically marked laboratory recipients (58).
302 IncHI2 plasmids were known to be associated with tellurite resistance determinants previously
303 (59, 60). Similarly, the *terC* gene was also detected on the largest IncHI2 plasmid in this study.
304 Cross-resistance mechanism typically involved common efflux pump systems which pumped out
305 structurally distinct agents/compounds such as metals and antibiotics (55, 56). Cheng et al.
306 showed that chromosomally encoded TetA(L) efflux pump was able to remove both tetracycline
307 and heavy metal cobalt (61). The *tet(A)* gene encoding major facilitator superfamily multidrug
308 efflux pump was detected in several unrelated plasmids in this study including IncHI2, IncN,
309 IncY, IncFIB(K) and IncI1 plasmids.

310 Prophage are normally found in bacterial chromosome, in particular within pathogenicity
311 islands in pathogens (18, 62). A mounting number of studies showed that bacteriophages
312 contributed to the widespread dissemination of antibiotic resistance genes via phage-mediated
313 transduction (63, 64). In this study, PHASTER tool was used to investigate how likely prophage
314 sequence could be detected on multi-drug resistant plasmids. Prophage regions were detected in
315 a majority of input plasmids; however, the hit scores were pretty low, suggesting these prophages
316 were unlikely to become active phages. The largest plasmid pT525A and the IncY plasmid
317 pT425A had better scores for prophage hits. There was a possibility that phage was able to insert
318 its sequences into plasmids, resulting in plasmids of larger size and more diversity as
319 exemplified by plasmid pT525A. IncY plasmids were known to be phage-like plasmids because

320 they shared a large portion of homologous segments with bacteriophage, in particular phage P1
321 (17, 19, 24, 65, 66).

322 In conclusion, this study genotypically characterized ESBL plasmids from dairy manure
323 including plasmid sizes, antibiotic resistance genes, incompatibility groups, toxin-antitoxin
324 systems, virulence factor and prophage regions. Sequencing a larger set of plasmids revealed
325 more distinct less frequent plasmids, especially in digested manure. The bla_{IMP-27} gene conferring
326 resistance to both carbapenem and third-generation cephalosporis was found integrated into the
327 host chromosome. The study provided some insights into the dynamics of ESBL genes and
328 plasmids carrying these genes in dairy manure.
329

MATERIALS AND METHODS

1. Conjugation method

The experiment was described in a previous study (25). Briefly, dairy manures were enriched with cefotaxime (4 mg/L) and then mated with *gfp*-labelled *E. coli* CV601 overnight. Transconjugants were selected on chromocult media containing rifampicin (50 mg/L), kanamycin (50 mg/L) and cefotaxime (4 mg/L).

2. Illumina/MinION sequencing protocol

3. Annotation tools to detect antibiotic resistance genes, mobile genetic elements and toxin-antitoxin systems

Antibiotic resistance genes were detected using starAMR tool (Galaxy Version 0.7.1+galaxy1) which searched Illumina short-read assemblies against the resfinder resistance gene database. Mobile genetic elements were detected by RAST (https://rast.nmpdr.org), and then subsequently specified by blasting sequences against the NCBI non-redundant database. RAST was also used to detect toxin-antitoxin systems on complete closed plasmids.

4. Detection of virulence factors

Complete closed plasmid sequences from hybrid assembly were used as input to VirulenceFinder 2.0, a web-tool (https://cge.cbs.dtu.dk/services/VirulenceFinder/), to detect virulence genes (67).

5. Detection of prophage sequences

A web-tool PHASTER (http://phaster.ca/) was used to identify and annotate prophage sequences within complete closed plasmids (68, 69).

6. Other tools used to construct plasmid maps and detect SNPs

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Table 1: Distribution of eighty-three sequenced plasmids based on their incompatibility groups, ESBL genes and other resistance genes

ESBL gene	Representative plasmid	Other resistance genes co-locating on the same plasmid	Incompatibility group	Size	Farm	Raw (n = 35)	Digested (n = 48)
bla$_{CTX-M-1}$	pT115A	None	IncN	42,592	1,2,5,7	10	8
bla$_{CTX-M-1}$	pT39A	*sul2, tet(A)*	IncI1	~75,251	3	NA	1
bla$_{CTX-M-14}$	pT428A	None	IncI1	91,905	2,3	3	NA
bla$_{CTX-M-14}$	pT593A	None	IncB/O/K/Z	~ 89,017	3	NA	1
bla$_{CTX-M-14b}$	pT59A	*aac(6')-Ib-cr, aph(3'')-Ib, ARR-3,dfrA27, qnrS3, sul1, sul2, tet(A)*	IncFIB(K)		5	NA	1
bla$_{CTX-M-14b}$	pT58A	*aadA16, ARR-3, bla*$_{TEM-1B,}$ *qnrS1, sul1, sul2, tet(A)*	IncFIB(K)		5	NA	1
bla$_{CTX-M-15}$	pT145A	None	IncI1	~ 85,051	1	2	NA
bla$_{CTX-M-15}$	pT415A	*aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, dfrA14, qnrS1, sul2, tet(A)*	IncY	~ 85,052	5	NA	1
bla$_{CTX-M-15}$	pT545A	*aac(6')-Ib-cr, aph(3'')-Ib, aph(6)-Id, bla*$_{OXA-1}$*, catB4, dfrA1, floR, lnu(G), sul2*	NA	104,875	1,2,3,4,5,7	17	22
bla$_{CTX-M-27}$	pT455A	None	IncN	42,273	2	NA	1
bla$_{CTX-M-27}$	pT257A	None	IncFIIA,IncFII	66,581	2	NA	2
bla$_{CTX-M-27}$	pT570A	*aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, dfrA14, sul2, tet(A)*	IncN	53,066	2	NA	3
bla$_{CTX-M-27}$	pT295A	*aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, dfrA14, sul2, tet(A)*	IncN, IncX1	77,311	2	NA	1
bla$_{CTX-M-55}$	pT525A	*aac(3)-IId, aadA22, aph(3')-Ia, ARR-2, bla*$_{TEM-1B}$*, dfrA14, floR, lnu(F), mph(A), qnrS1, sul3, tet(A)*	IncHI2, IncHI2A	266,763	3	2	NA
bla$_{CTX-M-55}$	pT588A	*aac(3)-IId, aadA22, aph(3')-Ia, ARR-2, bla*$_{TEM-1B}$*, dfrA14, floR, lnu(F), mph(A), qnrS1, sul3, tet(A)*	IncHI2, IncHI2A, IncN	~ 227,236	7	NA	1
bla$_{CTX-M-55}$	pT476A	*bla*$_{TEM-1B}$	IncX1	~ 40,307	7	NA	1
bla$_{CTX-M-55}$	pT156A	*bla*$_{TEM-206}$	IncFII(pHN7A8)	66,894	1	NA	1
bla$_{CTX-M-55}$	pT224A	*bla* $_{TEM-206}$*, fosA3*	IncFII(pHN7A8)	~ 70,625	7	NA	2
bla$_{PER-1}$	pT413A	*aadA2, aph(3')-Ia, mph(E), msr(E), sul1, sul1, tet(C), tet(E), tet(X)*	IncC	187,012	5	1	NA
bla$_{IMP-27}$	pT598A	*aph(6)-Id, strA, sul2, tet(A)*	NA	41,847	4	NA	1

544 Table 2. List of toxin-antitoxin systems found on plasmids

Plasmid ID	Toxin	Antitoxin	Type
pT82A	MazF	MazE	2
pT101A	MazF	MazE	2
pT156A	PemK	PemI	2
pT159A	MazE	MazF	2
pT209A	MazF	MazE	2
pT224A	PemK	PemI	2
pT257A	PemK	PemI	2
pT267A	MazF	MazE	2
pT270A	MazF	MazE	2
pT100A	YdcE	YdcD	2
pT390A	MazF	MazE	2
pT525A	HigB1, HipA	HipB	2
pT545A	MazF	MazE	2
pT593A	YdcE	YdcD	2
pT295A	RelE	RelB	2
pT413A	HigB	HigA	2

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Plasmid ID	Virulence factor	Identity	Query / Template length	Position in contig	Protein function	Accession number
pT100A	*cib*	100	1881 / 1881	6021..7901	Colicin ib	KP198616
pT428l	*cia*	100	1881 / 1881	5247..7127	Colicin ia	UDDL01000017

546 Table 3. Detection of virulence factors on plasmids

pT593A	*traT*	100	627 / 627	51274..51900	Outer membrane protein complement resistance	MF156268
pT525A	*terC*	100	1041 / 1041	78902..79942	Tellurium ion resistance protein	KU341381
pT156A	*traT*	100	732 / 732	53990..54721	Outer membrane protein complement resistance	CYCV01000028
pT224A	*traT*	100	734 / 735	56998..57732	Outer membrane protein complement resistance	NC_019073
pT257A	*traT*	100	735 / 735	52276..53010	Outer membrane protein complement resistance	NZ_CP032205
pT247A	*cia*	100	1881 / 1881	5247..7127	Colicin ia	UDDL01000017

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549 Table 4. Identification of putative prophages on plasmids by PHASTER

Plasmid ID	Region	Region Length	Completeness	# Total Proteins	Region Position	Most Common Phage	GC %
pT82A	1	7.5Kb	incomplete	12	24841-32401	PHAGE_Cronob_ENT39118_NC_019934(2)	41.86%
	2	33.7Kb	incomplete	12	23467-57208	PHAGE_Escher_RCS47_NC_042128(3)	49.47%
	3	4.9Kb	incomplete	7	51634-56533	PHAGE_Mycoba_Shipwreck_NC_031261(1)	51.49%
pT100A	1	20.5Kb	incomplete	17	13759-34330	PHAGE_Klebsi_phiKO2_NC_005857(2)	54.96%
	2	9.1Kb	incomplete	14	26925-36113	PHAGE_Entero_N15_NC_001901(2)	56.72%
pT101A	1	7Kb	incomplete	12	5797-12863	PHAGE_Escher_RCS47_NC_042128(3)	53.81%
	2	4.9Kb	incomplete	7	20935-25834	PHAGE_Mycoba_Shipwreck_NC_031261(1)	51.49%
pT115A	1	11.6Kb	incomplete	12	1-11645	PHAGE_Escher_RCS47_NC_042128(3)	51.70%
pT156A	1	15.4Kb	incomplete	12	2525-17988	PHAGE_Escher_RCS47_NC_042128(4)	50.37%
pT159A	1	15.3Kb	incomplete	26	310-15624	PHAGE_Escher_RCS47_NC_042128(3)	55.93%
pT199A	1	11.6Kb	incomplete	13	1-11638	PHAGE_Escher_RCS47_NC_042128(3)	51.70%
pT209A	1	15.3Kb	incomplete	26	266-15632	PHAGE_Escher_RCS47_NC_042128(3)	55.99%
pT224A	1	17.9Kb	incomplete	13	2525-20477	PHAGE_Escher_RCS47_NC_042128(3)	51.06%
pT247A	1	20.5Kb	incomplete	17	9428-29984	PHAGE_Klebsi_phiKO2_NC_005857(2)	55.10%
	2	9.1Kb	incomplete	14	22591-31767	PHAGE_Entero_N15_NC_001901(2)	56.61%
pT257A	1	13.3Kb	incomplete	8	2525-15842	PHAGE_Escher_RCS47_NC_042128(4)	50.74%
pT267A	1	15.3Kb	incomplete	26	266-15632	PHAGE_Escher_RCS47_NC_042128(3)	55.99%
pT270A	1	7.1Kb	incomplete	12	5753-12871	PHAGE_Escher_RCS47_NC_042128(3)	53.95%
	2	4.9Kb	incomplete	7	20943-25842	PHAGE_Mycoba_Shipwreck_NC_031261(1)	51.49%
pT295A	1	27.6Kb	incomplete	21	3066-30726	PHAGE_Escher_RCS47_NC_042128(3)	48.94%
pT390A	1	7.5Kb	incomplete	13	52679-60238	PHAGE_Cronob_ENT39118_NC_019934(2)	41.87%
	2	33.7Kb	incomplete	12	51310-85093	PHAGE_Escher_RCS47_NC_042128(3)	49.52%
	3	4.9Kb	incomplete	7	79519-84418	PHAGE_Mycoba_Shipwreck_NC_031261(1)	51.49%

pT428Al	1	20.5Kb	incomplete	17	9428-29984	PHAGE_Klebsi_phiKO2_NC_005857(2)	55.10%
	2	9.1Kb	incomplete	14	22591-31767	PHAGE_Entero_N15_NC_001901(2)	56.61%
pT415A	1	16.6Kb	questionable	20	1146-17798	PHAGE_Salmon_SJ46_NC_031129(3)	49.94%
pT455A	1	9.3Kb	incomplete	10	20175-29551	PHAGE_Salmon_SJ46_NC_031129(4)	53.36%
pT525A	1	41Kb	questionable	51	73364-114387	PHAGE_Acinet_vB_AbaM_ME3_NC_041884(3)	50.45%
	2	29.9Kb	questionable	25	142809-172780	PHAGE_Escher_RCS47_NC_042128(3)	52.21%
pT526A	1	24.6Kb	incomplete	9	7334-31962	PHAGE_Salmon_SJ46_NC_031129(4)	53.01%
pT545A	1	7.6Kb	incomplete	12	391-7990	PHAGE_Cronob_ENT39118_NC_019934(2)	41.96%
	2	4.9Kb	incomplete	7	81134-86033	PHAGE_Mycoba_Shipwreck_NC_031261(1)	51.49%
	3	7Kb	incomplete	12	94105-101171	PHAGE_Escher_RCS47_NC_042128(3)	53.81%
pT570A	1	24.6Kb	incomplete	9	7371-31999	PHAGE_Salmon_SJ46_NC_031129(4)	53.01%
pT593A	1	23.2Kb	incomplete	14	1517-24805	PHAGE_Escher_RCS47_NC_042128(3)	53.30%
	2	6.4Kb	incomplete	13	21185-27638	PHAGE_Entero_N15_NC_001901(2)	55.38%

The completeness was determined by PHASTER based on each hit score: Intact (score > 90), Questionable (score 70-90), Incomplete (score < 70).

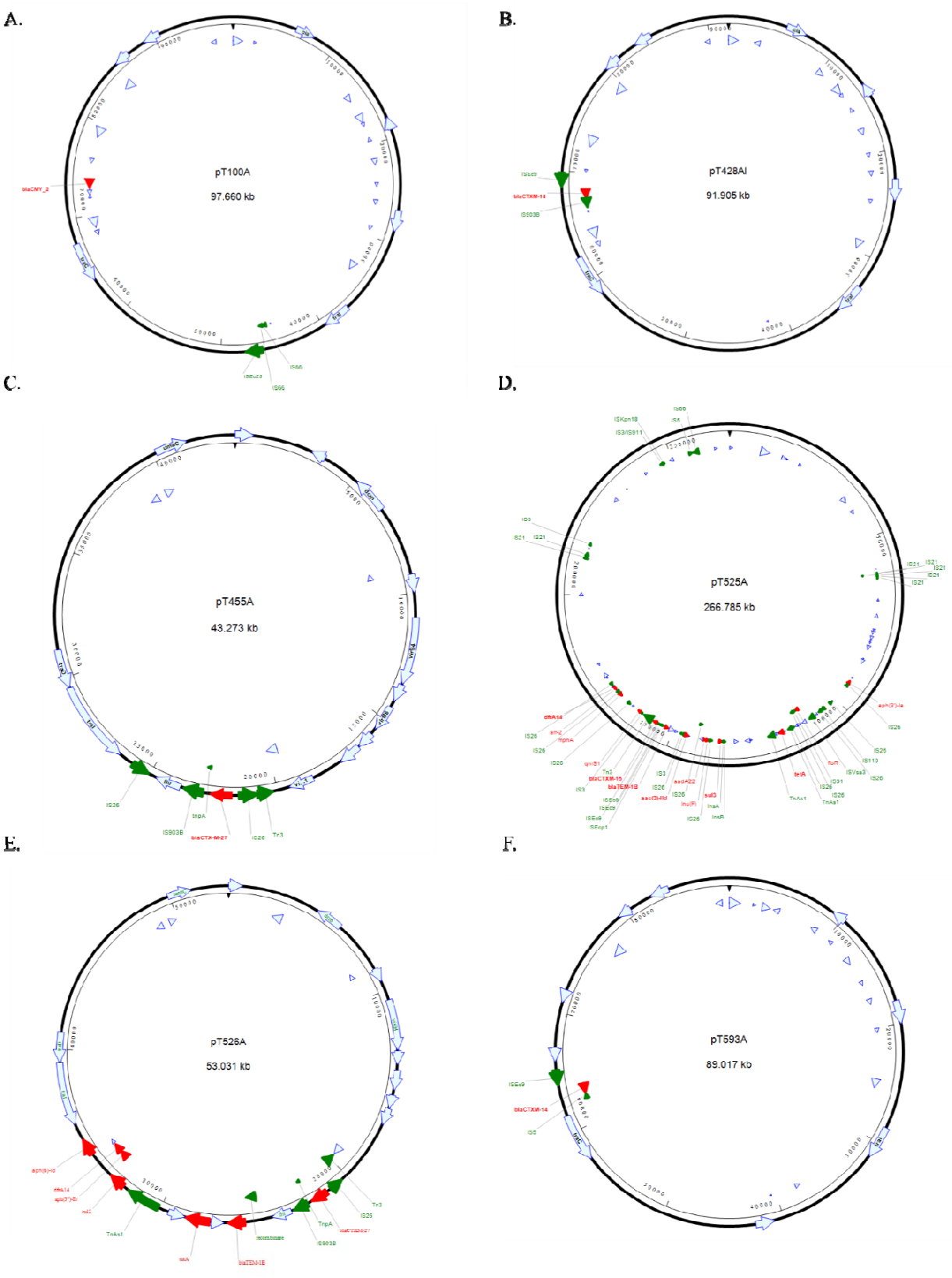

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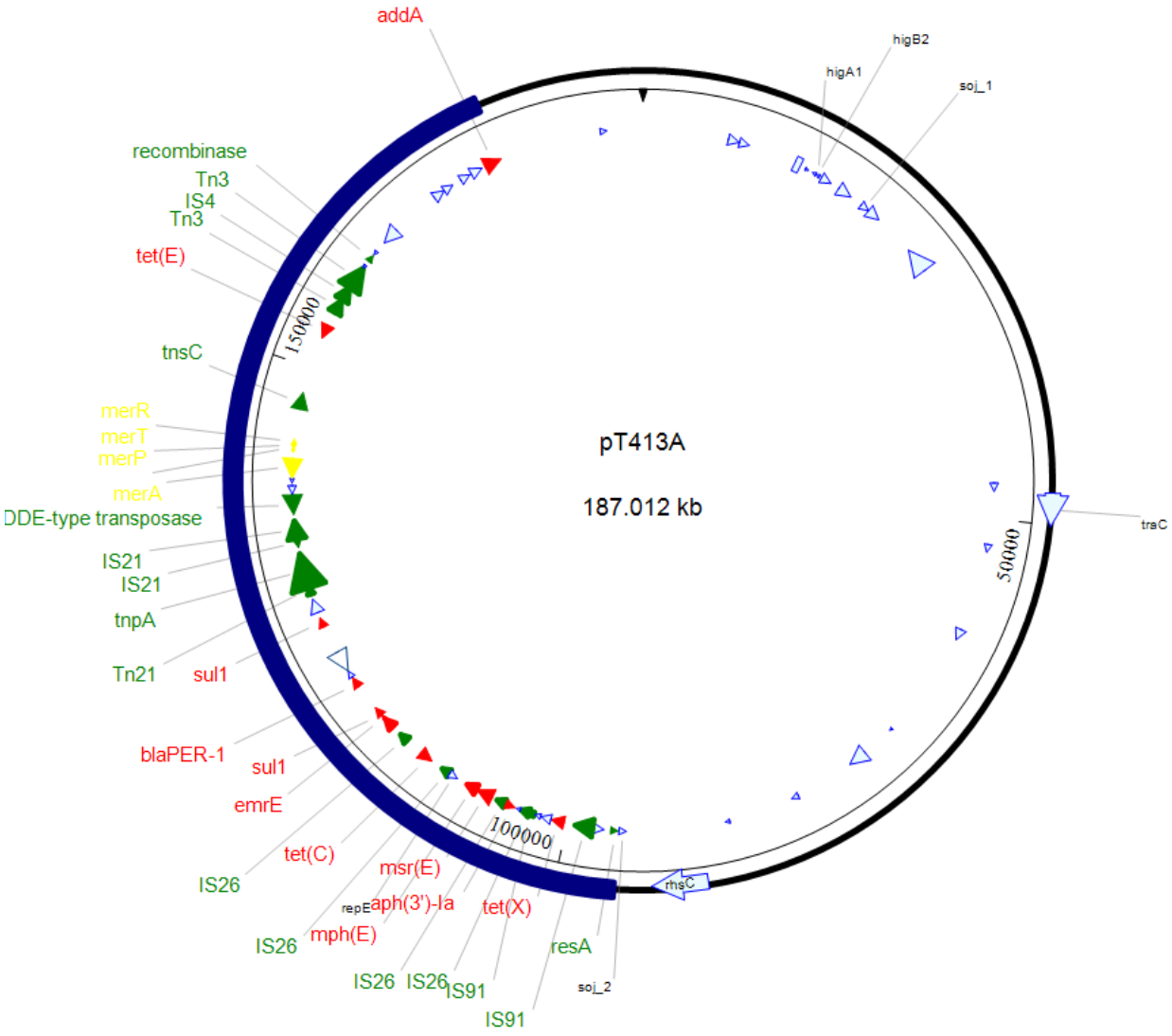
Fig. 1: Plasmid maps of six distinct plasmids harboring ESBL/AmpC genes which were captured in *E. coli* CV601 strain. (A) pT100A, (B) pT428Al, (C) pT455A, (D) pT525A, (E) pT526A, (F) pT593A. Red arrows are resistance genes detected by starAMR tool. Green arrows are mobile genetic elements detected by RAST and BLAST tools. Dark blue arrows are other functional genes which were annotated by PROKKA tool.



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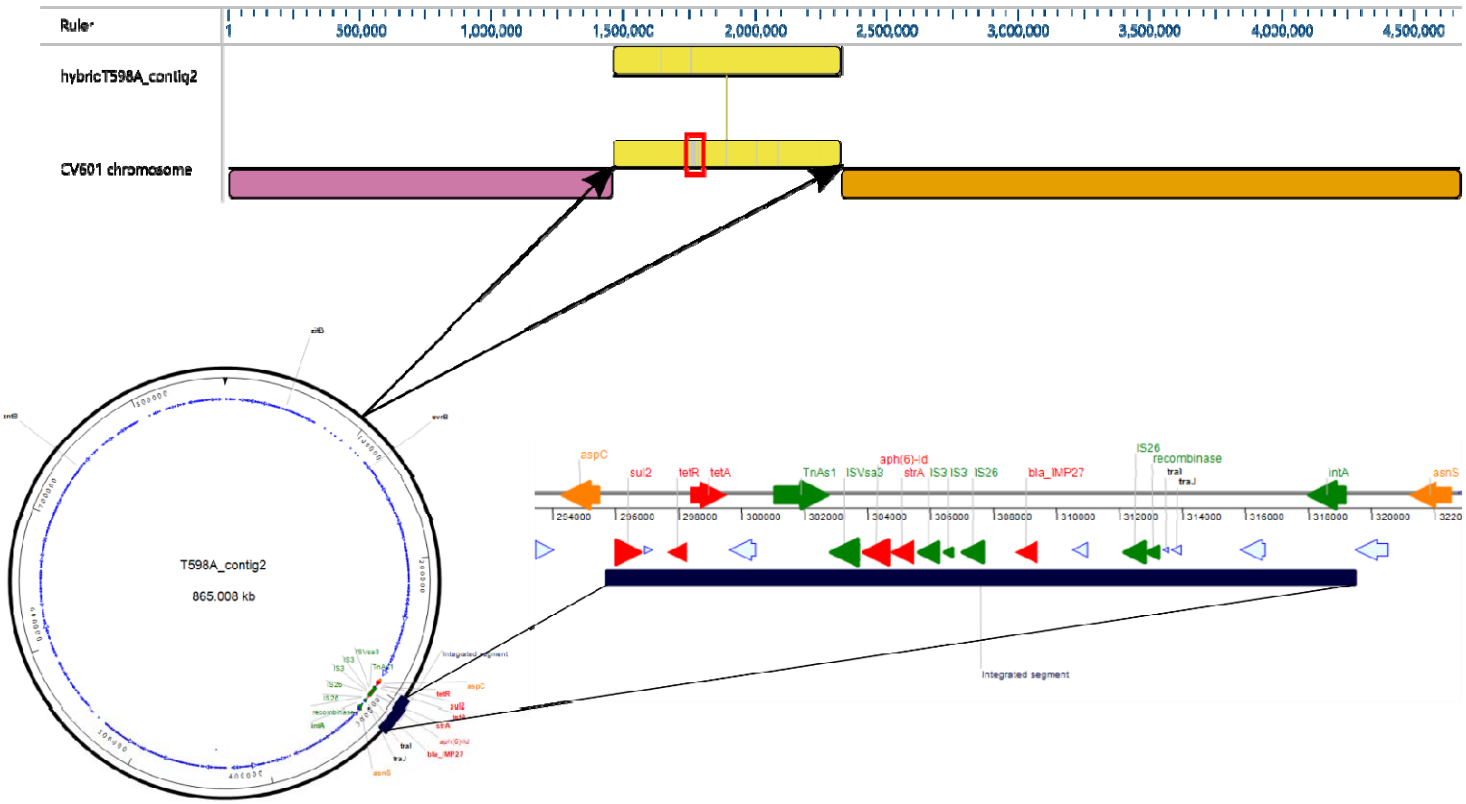
565

566 Fig. 2: The map of plasmid pT413A (187 kb). The blue stripe indicates the 79 kb-region
567 harboring mobile genetic elements (green arrows and labels), mercury resistance operon (yellow
568 arrows and labels) and antibiotic resistance genes (red arrows and labels).



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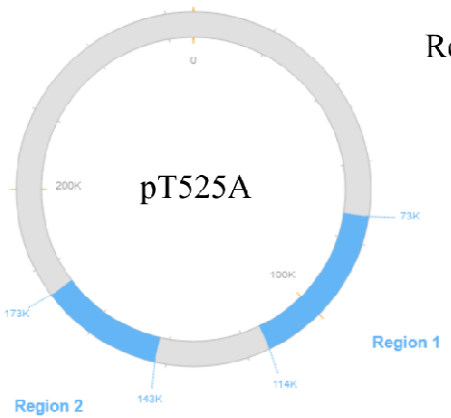
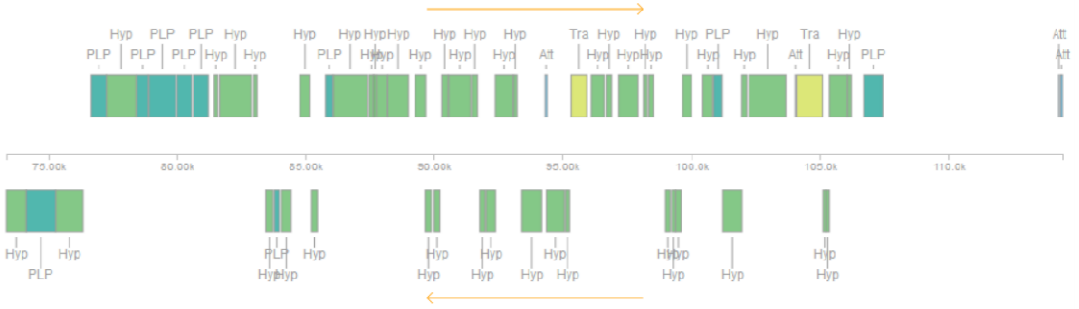
570

571 Fig. 3: Map of an integrated region containing multiple mobile genetic elements and resistance genes including the metallo-β-
572 lactamase gene *bla*$_{\text{IMP-27}}$. The top figure shows the alignment of the contig #2 of the isolate T598A with the host chromosome *E. coli*
573 CV601; the red box indicates the location where this *bla*$_{\text{IMP-27}}$-bearing region got integrated. The bottom left figure shows the circular

form of contig #2 with the integrated region. The bottom right figure is the enlarged integrated region. Red labels/arrows are resistance genes; green labels/arrows are mobile genetic elements (transposon, insertion sequence, integron); orange labels/arrows are genes on the host chromosome adjacent to the integrated region; blue arrows are other functional genes.



Region 1



Region 2

IncY plasmid
pT415A

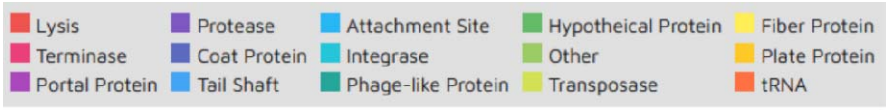


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Fig. 4: Map of putative prophage regions that were detected on plasmid pT525A (Region 1 & Region 2) and on IncY plasmid pT415A by PHASTER