Global molecular epidemiology of IMP-producing Enterobacteriaceae

Yasufumi Matsumura[a,b], Gisele Peirano[c,d], Mary R. Motyl[e], Mark D. Adams[f], Liang Chen[g], Barry Kreiswirth[g*], Rebekah DeVinney[a*] and Johann D.D. Pitout[a,c,d,h*#]

Departments of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada[a]; Department of Clinical Laboratory Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan[b]; Departments of Pathology & Laboratory Medicine[c], University of Calgary, Calgary, Alberta, Canada; Division of Microbiology, Calgary Laboratory Services, Calgary, Alberta, Canada[d]; Merck & Co., Inc., Rahway, NJ, USA[e]; J. Craig Venter Institute, La Jolla, CA, USA[f]; Public Research Institute TB Center, New Jersey Medical School, Rutgers University, Newark, NJ, USA[g]; Department of Medical Microbiology, University of Pretoria, Pretoria, South Africa[h]

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*Senior co-authors

#Address correspondence to Johann D.D. Pitout, johann.pitout@cls.ab.ca

Mark D. Adams's current address: The Jackson Laboratory for Genomic Medicine, Farmington, CT, USA

Abstract

International data on the molecular epidemiology of Enterobacteriaceae with IMP carbapenemases are lacking. We performed short read (Illumina) whole genomic sequencing on a global collection of 38 IMP-producing clinical Enterobacteriaceae (2008-14). IMP-producing Enterobacteriaceae (7 varieties within 11 class 1 integrons) were mainly present in South Pacific and Asia. Specific *bla*$_{IMP}$ containing integrons (In*809* with *bla*$_{IMP-4}$, In*722* with *bla*$_{IMP-6}$, In*687* with *bla*$_{IMP-14}$) were circulating among different bacteria in countries such as Australia, Japan and Thailand. In*1312* with *bla*$_{IMP-1}$ was present in *K. pneumoniae* from Japan and *C. freundii* from Brazil. *Klebsiella pneumoniae* (n=22) was the most common species; clonal complex (CC) 14 from the Philippines and Japan was the most common clone and contained In*1310* with *bla*$_{IMP-26}$ and In*1321* with *bla*$_{IMP-6}$. *Enterobacter cloacae* complex (n=9) consisted of *E. hormaechei* and *E. cloacae* cluster III. CC78 (from Taiwan) containing In*73* with *bla*$_{IMP-8}$, was the most common clone among *E. cloacae* complex. This study highlights the importance of surveillance programs using the latest molecular techniques in providing insight into the characteristics and global distribution of Enterobacteriaceae with *bla*$_{IMPs.}$

Introduction

Carbapenems are often the last line of effective therapy available for the treatment of serious infections due to multidrug-resistant bacteria. The rapid evolution of carbapenem resistance in Enterobacteriaceae during the last decade is an emerging global threat (1, 2), Enzymes that hydrolyze the carbapenems, known as carbapenemases, are the most important causes of carbapenem resistance. Carbapenemase-producing Enterobacteriaceae (CPE) have acquired multiple resistance genes making therapy of infections due to these bacteria challenging (1, 2).

The most common carbapenemases among CPE are KPCs (Amber class A), IMPs, VIMs, NDMs (class B or metallo-β-lactamases), and OXA-48-like (class D) enzymes (1). Metallo-β-lactamases (MBLs) hydrolyse all β-lactams except aztreonam although resistance levels may vary according to different subtypes. After the initial discovery of IMP-1 in Japan during 1991, bacteria with IMP enzymes have been detected worldwide (1). IMPs are common among MBL-*Pseudomonas aeruginosa* but remain relatively rare among members of the Enterobacteriaceae (3). IMP-producing Enterobacteriaceae are mainly identified in Asia-Pacific, (i.e. China, Japan, Taiwan, and Australia) (1, 4). The most common species associated with IMPs among the Enterobacteriaceae include *Klebsiella pneumoniae, Escherichia coli*, and *Enterobacter* spp (2, 3). IMP genes are often situated within class 1 integrons harboured on broad-host range plasmids, the exception being some bla_{IMP}-encoding class 2 and 3 integrons (2, 3). These mobile genetic elements play an important role in the interspecies distribution of IMP types of carbapenemases (5).

Comprehensive global data regarding the molecular epidemiology of CPE with bla_{IMP} are currently lacking. We designed a study that utilized short read whole genome sequencing to describe the molecular characteristics and international distribution of bla_{IMP} among Enterobacteriaceae obtained from the SMART global surveillance system.

Materials and methods

Bacterial isolates

We included 38 IMP-producing clinical, non-repeat Enterobacteriaceae collected from a global surveillance programs namely the Merck Study for Monitoring Antimicrobial Resistance Trends (SMART) [2008–2014] [Dataset 1]. The SMART program included isolates from intra-abdominal and urinary tract infections. The program collected consecutive clinically relevant gram-negative aerobes in each institution. These isolates initially underwent micro-dilution panel susceptibility testing and molecular screening for bla_{IMP} as described previously (6). Overall 107 366 isolates were obtained from 2008-14; of these 755 were positive for bla_{KPC}, 281 for $bla_{\text{OX-48-like}}$, 271 for bla_{NDM}, 89 for bla_{VIM} and 38 for $bla_{\text{IMP.}}$

Whole genome sequencing

We used the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) to prepare libraries for sequencing. Samples were multiplexed and sequenced on an Illumina NextSeq500 for 300 cycles (151 bp paired-end).

Genomic analysis

Draft genomes were obtained using SPAdes version 3.8.1 (7). Species identification was performed using SILVA 16s rRNA gene database release 123 (8). In addition, we used *hsp60* gene for identification of *Enterobacter* spp. (9) and whole genome-based phylogenetic tree including type strains for identification of *Klebsiella* spp. and *Citrobacter* spp. Average nucleotide identity (ANI) was calculated using JSpecies (10).

To define presence of resistance genes other than β-lactamases, plasmid replicons, and virulence genes of *Klebsiella* spp. and *E. coli* (detailed below), we used raw sequence data and SRST2 (11) (default settings: thresholds for detection of 90% identity and 90% coverage) in combination with ARG-ANNOT (12) and PlasmidFinder (13) databases. To perform MLST and to define presence of β-lactamases and other genes of interest, we used draft genomes and BLAST+ (14) in combination with following databases or typing schemes:

NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast/), NCBI Beta-Lactamase Data Resources (http://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/), plasmid addiction systems (15), and MLST (http://bigsdb.pasteur.fr/klebsiella/, http://pubmlst.org/ecloacae/, http://pubmlst.org/cfreundii/, http://mlst.ucc.ie/mlst/dbs/Ecoli/). We used BLAST+ thresholds of 90% nucleotide identity and 90% coverage, except for detection of gene alleles; we used 100% identity (β-lactamases and *ompK35*/*K36* at protein level and the others at nucleotide level).

The goeBURST algorithm implemented in PHYLOViZ software (16) was used to demonstrate relationships between STs and to define the founder of a clonal complex (CC). We defined CCs at the single-locus variant level. We used the assembled contigs with bla_{IMP} to determine their genetic environments. Integrons were classified according to INTEGRALL (http://integrall.bio.ua.pt/) and promoters of gene cassettes were characterized according to a previous study (17). For *Klebsiella* isolates, we performed *in silico* detection of K capsular type based on *wzi* alleles (18), virulence genes (http://bigsdb.pasteur.fr/klebsiella), and promoters and coding sequences of *ompK35*/*K36* (19, 20). For *E. coli* isolates, we performed *in silico* phylogenetic grouping (21), virulence genotyping (22), O:H typing (23), *fimH* typing (24), and detection of *H*30Rx-status (25).

Phylogenetic analysis

We used a core genome SNP-based approach to create a phylogenetic tree for each Enterobacteriaceae genus. First, we made the reference genome-like pseudo-chromosomes that contained only SNPs. For study isolates and 6 reference strains downloaded from the NCBI database (Dataset 2) of which complete genomes were not available, SNPs were identified using trimmed reads mapping to a genus specific reference genome (Dataset 2) followed by GATK Best Practices workflow (26) and SAMtools (27) (depth of sequencing >10 and Phred-score >20). Complete genomes and draft genomes of which raw reads were not available on the NCBI database (Dataset 2) were aligned against the reference genome of

the genus using ProgressiveMauve to obtain pseudo-chromosomes that contained only SNPs (28). The SNP-only core genome was identified as the common blocks of >500bp to all of the study isolates. Maximum-likelihood tree was build using these core genomes and RAxML (29) and visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Accession numbers

We deposited the sequencing data in the DDBJ and NCBI databases (accession no. DRA004879 and SRP046977). The sequences of new integrons described in this study ranged from accession no's LC169564 to LC169589.

Results and Discussion

Geographic distribution showed IMP-producing Enterobacteriaceae mainly in Asia.

The 38 IMP-producing Enterobacteriaceae isolates were obtained from eight countries, mainly from South Pacific (n=21), Asia (n=15) and 1 each from Brazil and Spain (Fig. 1 and Dataset 1). The common sources were intra-abdominal specimens and urines (n=27 and 11, respectively). The isolates include the following species: *K. pneumoniae* (n=22), *Enterobacter cloacae* complex (n=9), *Citrobacter* spp. (n=4), *E. coli* (n=3) (Fig 1, Table 1).

The 38 genomes were sequenced at an average depth of 120 (standard deviation [SD] 56.3) (Dataset 1). Assembled genomes had an average number of contigs of 104 (SD 41.5) and N50 value of 283,649 bp (SD 107,564 bp). We confirmed the presence of bla_{IMP} in the draft genomes of all the isolates.

The presence of resistance genes, antibiotic resistance profiles, plasmid replicons, and plasmid addiction systems are shown in Fig. S1. Table 1 shows the geographical distribution of the different species, types of carbapenemases and integrons. We identified 7 bla_{IMP} variants namely: $bla_{\text{IMP-1}}$ (n=5), $bla_{\text{IMP-4}}$ (n=8), $bla_{\text{IMP-6}}$ (n=4), $bla_{\text{IMP-8}}$ (n=4), $bla_{\text{IMP-13}}$ (n=1), $bla_{\text{IMP-14}}$ (n=3), $bla_{\text{IMP-26}}$ (n=13). The following genes were present in more than 1

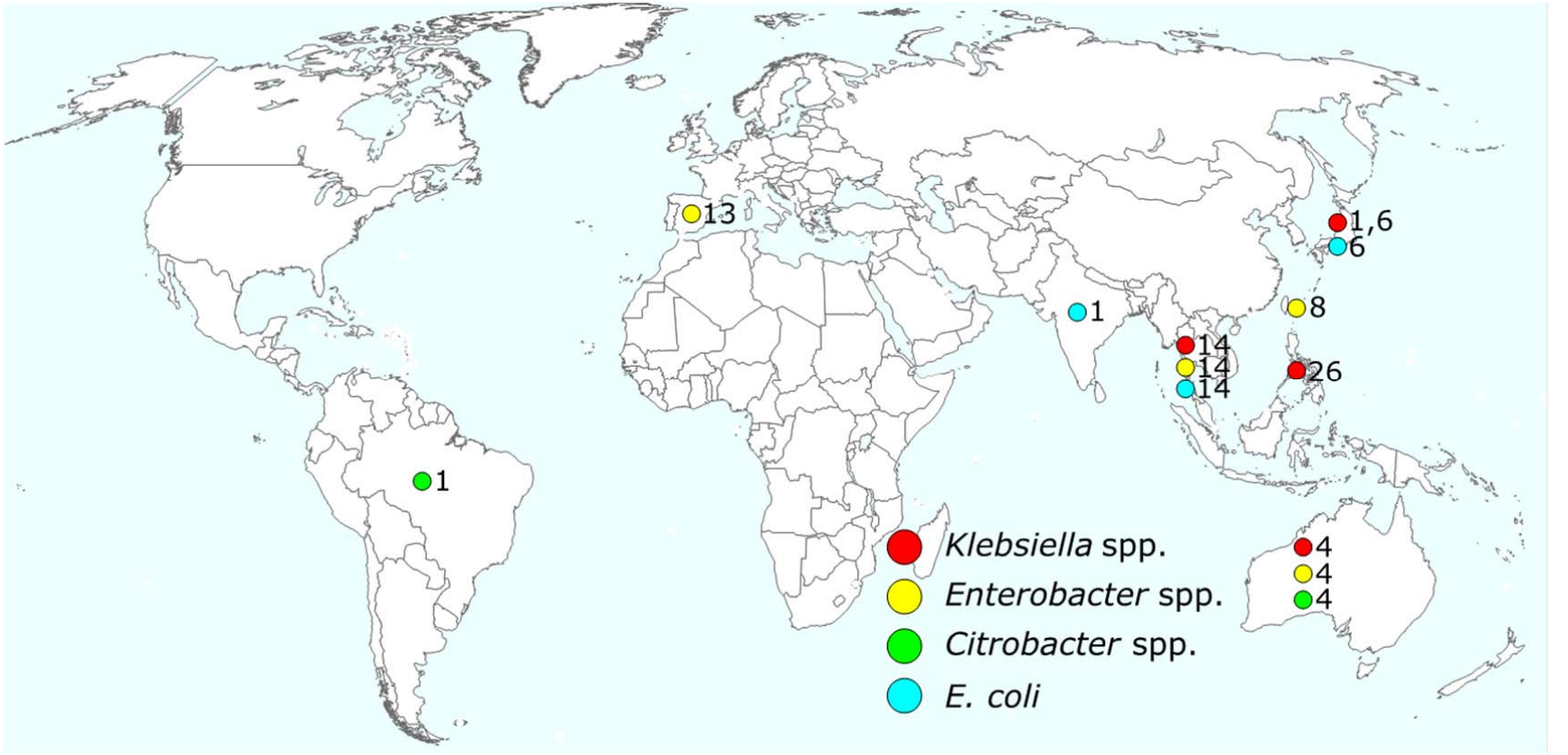

Fig. 1. Global distribution of IMP-producing Enterobacteriaceae isolates in this study.
Numbers beside circles indicate IMP subtypes.

Table 1. IMP subtypes and integrons of the Enterobacteriaceae isolates.

Carbapenemase (*n*)	Species, *n* (country)				
	Klebsiella spp. (KP)	*E. cloacae* complex (Ecl)	*Citrobacter* spp. (CI)	*E. coli* (EC)	Defined integron numbers (species, *n*)
IMP-1 (5)	3 (Japan)		1 (Brazil)	1 (India)	In*1312* (KP, 2; CI, 1), In*1311* (KP, 1), In*1313* (EC, 1)
IMP-4 (8)	2 (Australia)	3 (Australia)	3 (Australia)		In*809* (CI, 3; Ecl, 1)
IMP-6 (4)	3 (Japan)			1 (Japan)	In*722* (KP, 1; EC, 1), In*1321* (KP, 1)
IMP-8 (4)		4 (Taiwan)			In*73* (Ecl 3)
IMP-13 (1)		1 (Spain)			In*1319* (Ecl, 1)
IMP-14 (3)	1 (Thailand)	1 (Thailand)		1 (Thailand)	In*687* (KP, 1; EC, 1), In*1314* (Ecl, 1)
IMP-26 (13)	13 (Philippines)				In*1310* (KP, 6)

In*1310* to In*1314*, In*1319*, and In*1321* were novel integrons found in this study.

species: *bla*$_{\text{IMP-1}}$, *bla*$_{\text{IMP-4}}$, *bla*$_{\text{IMP-6}}$, and *bla*$_{\text{IMP-14}}$ (Table 1). Enterobacteriaceae with *bla*$_{\text{IMP-14}}$ (i.e. *K. pneumoniae. Enterobacter* spp. and *E. coli*) and *bla*$_{\text{IMP-13}}$ (*Enterobacter* spp.) were obtained from urines in Thailand and Spain; the remainder of *bla*$_{\text{IMPs}}$ were present in both urines and intra-abdominal specimens. The distribution of the different *bla*$_{\text{IMP}}$ subtypes was similar to previously published data (i.e. *bla*$_{\text{IMP-1}}$ and *bla*$_{\text{IMP-6}}$ were present in Japan, *bla*$_{\text{IMP-4}}$ in Australia, *bla*$_{\text{IMP-8}}$ in Taiwan, *bla*$_{\text{IMP-14}}$ in Thailand, and *bla*$_{\text{IMP-26}}$ in Philippines (Table 1) (2, 30-32).

Characterization of Class 1 integrons identified 11 different integron types including 7 novel cassette combinations.

All of the *bla*$_{\text{IMPs}}$ were situated within class 1 integrons. We were unable to sequence the complete integron-associated gene cassettes in 13 isolates due to the limitations associated with short-read sequencing.

We identified 11 different integron types containing *bla*$_{\text{IMP}}$ including 7 novel cassette combinations (Table 2). The novel cassette combinations included the following: *bla*$_{\text{IMP-26}}$*-qacG-aacA4* (In*1310*), *aacA4-bla*$_{\text{IMP-1}}$*-aadA2-tnpA* (In*1311*), *bla*$_{\text{IMP-1}}$*-aacA31-bla*$_{\text{OXA-142}}$ (In*1312*), *bla*$_{\text{OXA-10}}$, *aacA4-bla*$_{\text{IMP-1}}$*-qacG* (In*1313*), *bla*$_{\text{IMP-14}}$*-bla*$_{\text{OXA-10}}$, *aacA4* (In*1314*), *bla*$_{\text{IMP-13}}$*-aacA4-bla*$_{\text{OXA-2}}$ (In*1319*), *aacA4-bla*$_{\text{IMP-6}}$*-aadA2-tnpA* (In1321). In*1310* containing *bla*$_{\text{IMP-26}}$*-qacG-aacA4* was the most common cassette. The novel integron In*1312* with *bla*$_{\text{IMP-1}}$*-aacA31-bla*$_{\text{OXA-142}}$ had international, intercontinental, and inter-genus distribution (present in *K. pneumoniae* from Japan and *C. freundii* from Brazil). Country-specific *bla*$_{\text{IMP}}$ subtypes corresponded to the specific integron types previously characterized in that country (i.e. *bla*$_{\text{IMP-4}}$, In*809* from Australia (33); *bla*$_{\text{IMP-6}}$, In*722* from Japan (34); *bla*$_{\text{IMP-8}}$, In*73* from Philippines (35); *bla*$_{\text{IMP-14}}$, In*687* from Thailand (31).

The *aacA* variants (especially *aacA4*) aminoglycoside acetyltransferase genes were the most prevalent gene cassette in the different integron cassette combinations. They were

Table 2. Details of class 1 integrons with *bla*$_{IMP}$.

Integron number						Accession number of the In
Major type	Variant	*n*	Gene cassettes	Promoter type (*n*)	Downstream of gene cassettes (*n*)	
In*73*		3	*bla*$_{IMP-8}$-*aacA4-catB4*	PcH1 (1), UD (2)	*qacEΔ1-sul1*-IS*6100*[a] (1), UD (2)	AF322577
In*687*		2	*bla*$_{IMP-14}$-*aacA34*	UD (2)	UD (2)	LC169587
In*722*	In*722*	2	*aacA4-bla*$_{IMP-6}$-*aadA2-tnpA*[b]	PcH1 (2)	*qacEΔ1-sul1-orf5-orf6*-IS*6100* (1), *qacEΔ1-sul1*-Δ*orf5-chrA-padR*-IS*6100* (1)	AB616660
	In*1311*	1	*aacA4-bla*$_{IMP-1}$-*aadA2-tnpA*	PcH1 (1)	*qacEΔ1-sul1-orf5-orf6*-IS*6100* (1)	LC169566
	In*1321*	1	*aacA4-bla*$_{IMP-6}$-*aadA2-tnpA*[b]	PcH1 (1)	*qacEΔ1-sul1-orf5-orf6*-IS*6100* (1)	LC169589
In*809*		4	*bla*$_{IMP-4}$-*qacG-aacA4-catB4*	PcW$_{TGN-10}$ (2), PcH2$_{TGN-10}$ (1), UD (1)	*qacEΔ1-sul1*-Δ*orf5*-IS*CR1-sapA-orf2-qnrB2-pspF*-Δ*qacE*Δ*1-sul1-orf5*-IS*5075-chrA-padR*-IRt (1), *qacEΔ1-sul1*-Δ*orf5*-IS*4321R*-like-*chrA-padR*-IRt (1), UD (2)	JX101693
In*1310*		6	*bla*$_{IMP-26}$-*qacG-aacA4*	UD (6)	*qacEΔ1-sul1*-IS*CR1* (5), *qacEΔ1-sul1-orf5*-Δ*tniB-tniA*-IRt (1)	LC169564, LC169565

In*1312*	3	*bla*$_{\text{IMP-1}}$-*aacA31*-*bla*$_{\text{OXA-142}}$	PcH1 (2), PcS$_{\text{TGN-10}}$ (1)	*qacEΔ1*-*sul1*-*orf5*-*orf6*-IS*6100* (3)	LC169567
In*1313*	1	*bla*$_{\text{OXA-10}}$, *aacA4*-*bla*$_{\text{IMP-1}}$-*qacG*	PcW$_{\text{TGN-10}}$ (1)	*tniR*-*tniQ*-*tniB*-*tniA*-IRt (1)	LC169568
In*1314*	1	*bla*$_{\text{IMP-14}}$-*bla*$_{\text{OXA-10}}$, *aacA4*	PcW$_{\text{TGN-10}}$ (1)	UD (1)	LC169569
In*1319*	1	*bla*$_{\text{IMP-13}}$-*aacA4*-*bla*$_{\text{OXA-2}}$	PcH1 (1)	*qacEΔ1*-*sul1*-*orf5*-Δ*orf6*-IS*6100* (1)	LC169585

UD, undetermined due to a contig break in 5'-conserved segment (CS) or 3'-CS; IRt, inverted repeat of Tn*402*-like transposon.

[a] 97 bp of 5' side of 3'-CS was absent.

[b] In*722* and In*1321* have a different *aacA4* allele (*aacA4'-3* and *aacA4'-36*, respectively).

present in all 11 *bla*$_{IMP}$-containing integrons. The second common cassette was aminoglycoside adenylyltransferase gene (*aad* variants), which was present in 3 *bla*$_{IMP}$-containing integrons.

The *bla*$_{OXA-142}$ cassette had previously been identified in class 1 integrons among *P. aeruginosa* from Bulgaria (without *bla*$_{IMP}$) (36) and from Taiwan (with *bla*$_{IMP}$) (37). This suggests that appropriate surveillance and control methods may need to extend beyond the Enterobacteriaceae.

Integrons with weak promoters (i.e. PcW and PcH1) were common whereas strong promoters (i.e. PcS and PcH2) were rare (Tables 2, S1). There was no correlation with the type of promoters (weak vs strong) and MICs to ertapenem and imipenem. It seems that the carbapenem MIC are influenced by various factors, such as type of IMP, porin deficiency, presence of other extended-spectrum beta-lactamases, and efflux pumps. We were able to characterize the downstream structures in 9 *bla*$_{IMP}$-containing integrons (Tables 2, S2). All integrons contained 3'-CS structures immediately downstream of the gene cassettes except for *bla*$_{IMP-1}$-containing In*1313* with Tn*402*-like *tniRQBA* structure (38) (Table 2). The majority contained In*4*-like structures consisting of 3'-CS-IS*6100* (with or without partial deletion of 3'-CS and *chrA*-*padR* insertion).

The integron diversity observed in this study most likely represent the following: a) the sequential evolution over time of structurally similar cassettes (i.e. *bla*$_{IMP-1,}$ *bla*$_{IMP-6}$ with only one SNV difference) situated within homogenous integrons [i.e. variants of In*722*]. b) the multiple acquisition of the same IMP variant within more genetically divergent integron structures (e.g. *bla*$_{IMP-14}$ situated within In*687*, In*1314*).

K. pneumoniae subsp. _pneumoniae_ with two dominant clonal complexes

The phylogenetic relationships in Fig. 1 identified all of the 22 *K. pneumoniae* isolates as *K. pneumoniae* subsp. *pneumoniae*. *K. pneumoniae* isolates consisted of 6 CCs and 2 STs (Fig. 2). The most prevalent CCs (with ≥ 4 isolates) included CC14 [n=11] (from Japan

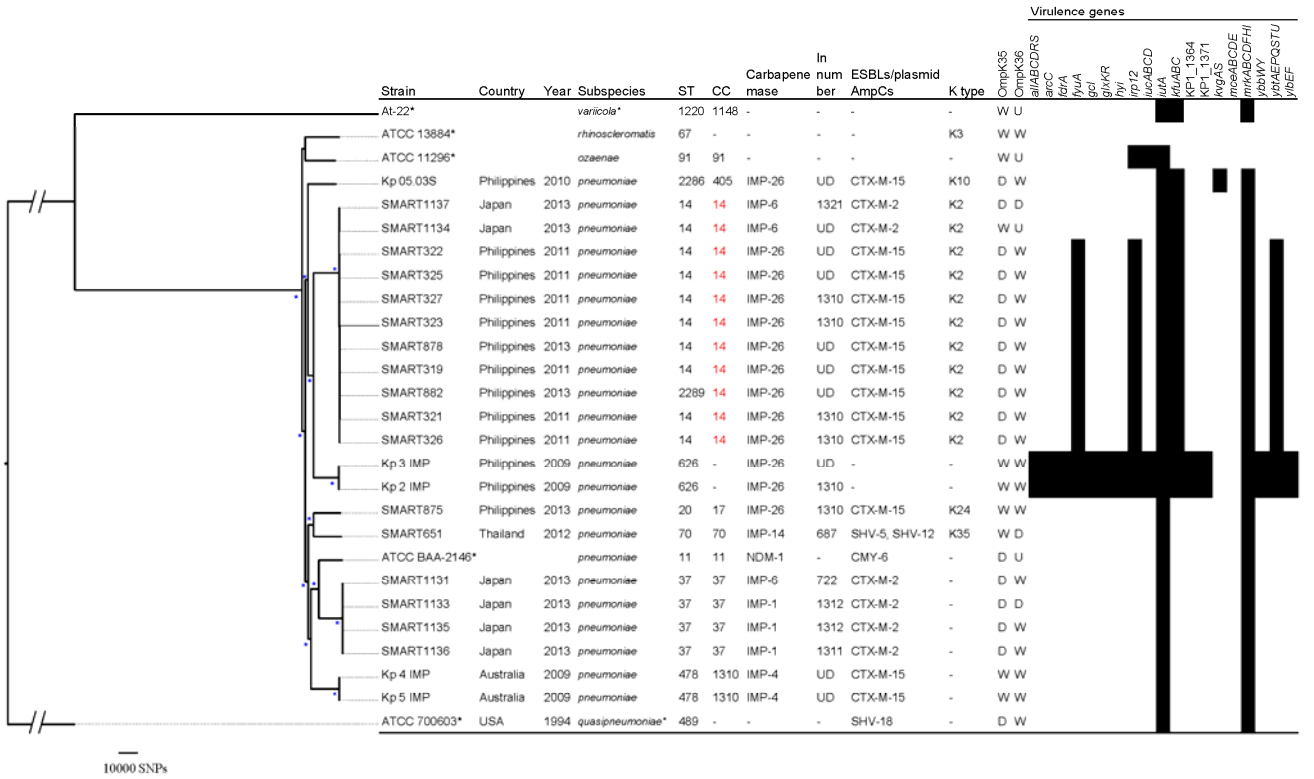

Fig. 2. Phylogenetic tree of IMP-producing *K. pneumoniae*. This maximum-likelihood phylogram is based on a 4,437,167 bp core genome and a total of 417,610 SNPs. The core genome was identified using *K. pnuemoniae* subsp. *pneumoniae* ATCC BAA-2146 as a reference genome. The tree included 22 study isolates and 5 reference strains (marked with asterisks). The tree is rooted by using the outgroup of *K. quasipneumoniae* ATCC700603 and asterisks indicates bootstrap support > 90% from 100 replicates. In the subspecies column, *K. variicola* and *K. quasipneumoniae* (marked with asterisks) are not subspecies of *K. pneumoniae* but distinct species. ST2286 and ST2289 were novel types found in this study. A CC marked with red color distributed internationally. OmpK35 and OmpK36 columns indicate predicted mutation of porins: W, wild; D, deficient (due to premature stop codon); V, variant associated with increased MIC of carbapenems; U, variant with unknown significance. Virulence genes of *clbA-R* (collibactin), *iroBCDN* (salmochelin), and *rmpA/rmpA2* were sought but not found.

and Philippines) and CC37 [n=4] (from Japan). In*1310* with bla_{IMP-26} from the Philippines and In*1321* with bla_{IMP-6} from Japan with were present in CC14. CC14 is the only clone with international distribution. ST14 and ST37 are global multidrug-resistant clones and had been associated with the production of AmpC β-lactamases, extended-spectrum β-lactamases (ESBLs) and carbapenemases (39, 40).

OmpK35 and OmpK36 deficiencies and variants are responsible for alterations in porins that contribute to increased MICs to the carbapenems (39). The majority of the study isolates had OmpK35 deficiency due to premature stop codons and wild-type OmpK36 (Fig. 2).

K1, K2, K5, K20, K54, and K57 capsular types are associated with community-acquired invasive infections due to *K. pneumoniae* (18). CC14 isolates from this study were positive for K2 and present in Japan and Philippines (Fig. 2). Brisse et al. reported that CC14-K2 was not associated with *rmpA* (i.e. regulator of mucoid phenotype) and mouse lethality when compared to CC65-K2 (41).

Hypervirulent *K. pneumoniae* strains often possess siderophore clusters (i.e. yersiniabaction, aerobactin, colibactin, and salmochelin) as well as *rmpA* or *rmpA2* (40). Yersiniabactin, which is encoded by a pathogenicity island that includes *ybt*, *irp12* and *fyuA* genes (40) was present in isolates from this study belonging to CC14 and ST626 (Fig. 2).

E. cloacae complex consisted of _E. hormaechei subsp. steigerwaltii_ and subsp. _oharae_, and _E. cloacae_ cluster III.

E. cloacae complex is made up of 13 groups which are difficult to distinguish using phenotypic methods (9). Recent studies showed that *E. hormaechei* and *E. cloacae* cluster III are the most prevalent clinical species among *E. cloacae* complex (42, 43). *E. hormaechei* subsp. *steigerwaltii* are the most prevalent sub-species, followed by subsp. *oharae* while subsp. *hormaechei* are generally rare (42, 43).

E. cloacae complex (n=9) was the second most common species in our study and

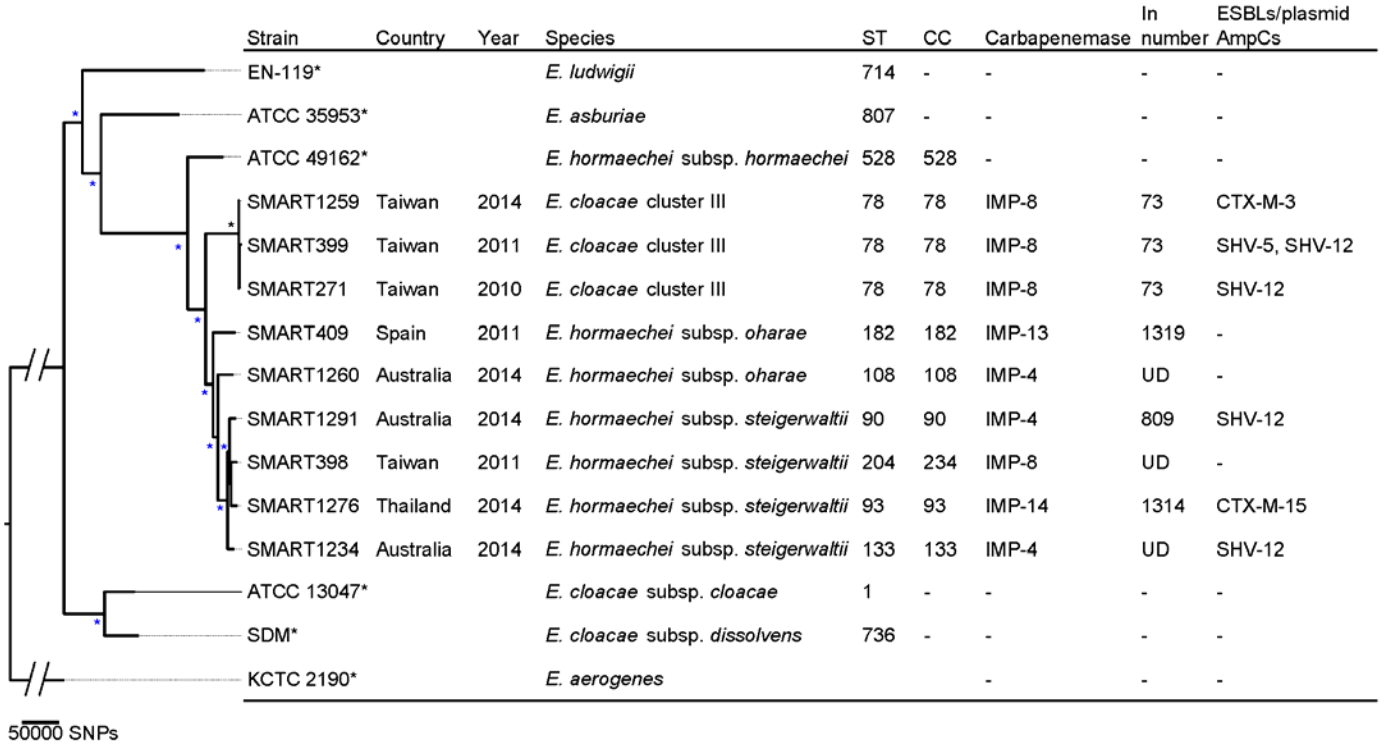

Fig. 3. Phylogenetic tree of IMP-producing *Enterobacter* spp. This maximum-likelihood phylogram is based on a 1,803,217 bp core genome and a total of 478,902 SNPs. The core genome was identified using *E. cloacae* subsp. *cloacae* ATCC 13047 as a reference genome. The tree included 9 study isolates and 6 reference strains (marked with asterisks). The tree is rooted by using the outgroup of *E. aerogene* s KCTC 2190 and asterisks indicates bootstrap support > 90% from 100 replicates. ST512, ST514, and ST520 were novel types found in this study.

consisted of *E. hormaechei* subsp. *steigerwaltii*, subsp. *oharae*, and *E. cloacae* cluster III (Fig. 3). *In-silico* MLST analysis identified 7 CCs among *E. cloacae* complex (Fig. 3). *E. cloacae* cluster III CC78 (with $bla_{\text{IMP-8}}$ from Taiwan) was the most common CC among *E. cloacae* complex. Previous molecular epidemiology studies have shown that CC78 are global clones associated with $bla_{\text{CTX-M-15}}$ or $bla_{\text{VIM-1}}$ especially among European countries (44).

Citrobacter* spp. and *Escherichia coli

Citrobacter spp. isolates (n=4) included in our study belonged to STs 97 to 100 (Fig. 4). Two isolates (SMART316 and SMART314) were classified as *Citrobacter* spp. based on the phylogenetic tree constructed with type strains (Fig. 4) (45). The ANI values between these 2 isolates and the 5 most closely related *Citrobacter* species (i.e. *C. freundii*, *C. braakii*, *C. werkmanii*, *C. youngae*, and *C. pasteurii*) were < 95 percentage (i.e. is the cut-off value of species definition) [Table S3]. ANI is a promising method of defining species using whole genome sequencing replacing DNA-DNA hybridization (10).

The phylogenetic relationship of *E. coli* isolates (n=3) is shown in Fig. S2. Two of the *E. coli* isolates belonged to the multidrug-resistant ST131 pandemic clone, which has been associated with fluoroquinolone resistance and ESBLs, including the recent acquisition of carbapenemases (6). The $bla_{\text{IMP-14}}$ in one of the ST131 isolate (SMART640) was nested within a 54-kb multidrug resistance region located on an epidemic IncA/C2 plasmid (46).

This study has several limitations. Our collection may not represent the global prevalence of IMP and integrons subtypes. We were unable to determine all of the integron structures due to the limitation of short-read sequencing. Long-read sequencing techniques including the detailed analysis of plasmids would provide more knowledge on location, mobile elements, and plasmid backbones of these carbapenemases.

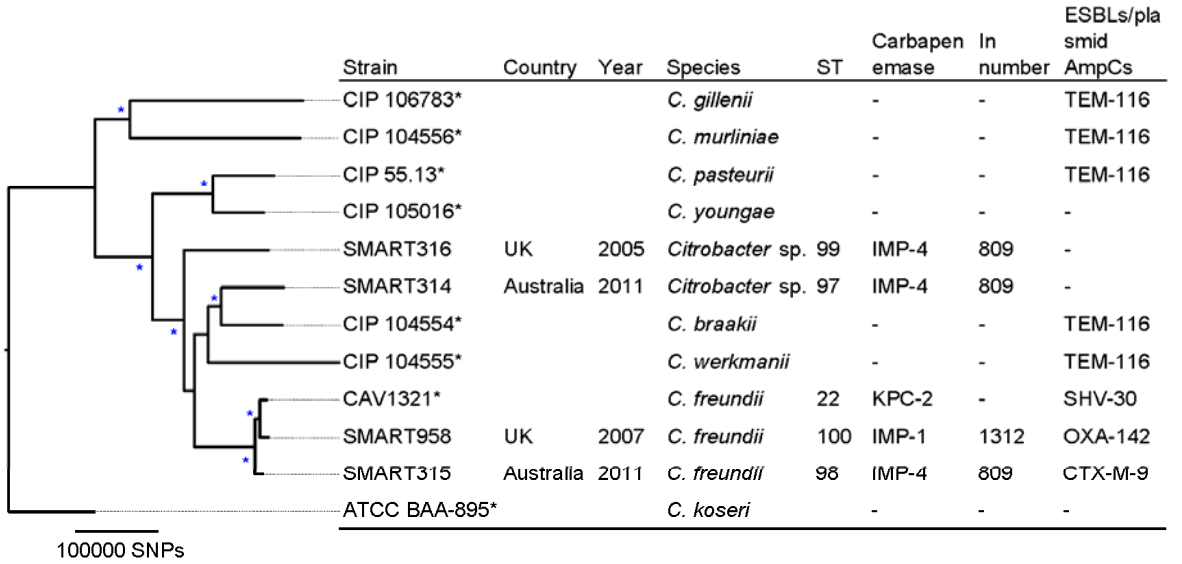

Fig. 4. Phylogenetic tree of IMP-producing *Citrobacter* spp. This maximum-likelihood phylogram is based on a 2,410,533 bp core genome and a total of 609,249 SNPs. The core genome was identified using *C. freundii* CAV1321 as a reference genome. The tree included 4 study isolates and 8 reference strains (marked with asterisks). The tree is rooted by using the outgroup of *C. koseri* ATCC BAA-895 and asterisks indicates bootstrap support > 90% from 100 replicates. STs 97 to 100 were novel types found in this study.

Summary

We used whole genome sequencing with comprehensive molecular analysis to elucidate the global epidemiology at a large scale of bla_{IMP}-containing Enterobacteriaceae and showed that some bla_{IMP} subtypes with associated integrons were present in certain countries within multiple species. Examples include the flowing: a) In*809* was present in *E. hormaechei* subsp. *steigerwaltii, C. freundii* and *Citrobacter* sp. from Australia; b) In*722* was present in *K. pneumoniae* and *E. coli* from Japan; c) In*687* was identified in *K. pneumoniae* and *E. coli* from Thailand; d) In*1312* was present in *K. pneumoniae* from Japan and *C. freundii* from Brazil. This study identified certain high-risk global clones with specific IMP integrons (i.e. *K. pneumoniae* ST14 from the Philippines contained In*1310*, *K. pneumoniae* ST37 from Japan contained In*722*, In*1311* and In*1312* while *E. cloacae* ST78 from Taiwan contained In*73*).

This study highlights the importance of surveillance programs using the latest molecular techniques in providing insight into the characteristics, global distribution of CCs and their association with integrons on containing bla_{IMPs}. Our results suggest that specific bla_{IMP} containing integrons are circulating locally among different bacteria in countries such as Australia, Japan and Thailand while the identification of high-risk clones has the potential to expand the global distribution of CPE. This emphasise the importance for identifying global types of IMPs among different Enterobacteriaceae species and clones.

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Transparency declaration

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Supplementary materials.

Table S1. Promoter types of class 1 integrons with *bla*$_{IMP}$.

Promoter type	No. of integron types (%)	No. of isolates (%)
PcS	0	0
PcS_{TGN-10}	1 (11%)	1 (7%)
PcH2	0	0
$PcH2_{TGN-10}$	1 (11%)	1 (7%)
PcW_{TGN-10}	3 (33%)	4 (29%)
PcW-P2	0	0
PcH1	6 (67%)	8 (57%)
$PcH1_{TTN-10}$	0	0
No. of types/isolates determined	9	14
Total no. of types/isolates	0	0

The denominators of percentages are calculated using numbers of types/isolates determined.

Promoter types were presented in order of promoter strength.

Table S2. Downstream of gene cassettes of class 1 integrons with *bla*$_{IMP}$.

Immediate downstream	No. of integron types (%)	No. of isolates (%)
3'-CS-like		
IS*CR1* insertion or IS*4321R* insertion variants of 3'-CS (*qacEΔ1-sul1-orf5*)-Δ*tniB-tniA*-IRt	2 (22%)	3 (17%)
3'CS (*qacEΔ1-sul1-orf5-orf6*)-IS*6100*[a]	6 (67%)	9 (50%)
3'CS (*qacEΔ1-sul1*)-IS*CR1*	1 (11%)	5 (28%)
Non-3'-CS		
Tn*402*-like *(tniRQBA*)	1 (11%)	1 (6%)
No. of types/isolates determined	9	18
Total no. of types/isolates	11	25

CS, conserved segment; IRt, inverted repeat of Tn*402*-like transposon.

The denominators of percentages are calculated using numbers of types/isolates determined.

[a] A part of 3'-CS (*orf6* with or without *orf5*) was deleted in 3 integron types (3 isolates). *chrA-padR* was inserted between a part of 3'-CS (*qacEΔ1-sul1-*Δ*orf5*) and IS*6100* in 1 integron type (1 isolates).

Table S3. Average nucleotide identity (ANI) of *Citrobacter* isolates that does not belong to any known species.

	Reference strain					Isolate	
	C. freundii	*C. braakii*	*C. werkmanii*	*C. youngae*	*C. pasteurii*	SMART316	SMART314
Isolate	CAV1321	CIP104554	CIP 104555	CIP 105016	CIP 55.13		
SMART316	92.34%	91.92%	89.71%	89.94%	91.92%	-	91.79%
SMART314	92.47%	93.12%	90.78%	89.38%	93.12%	91.72%	-
SMART315[a] (*C. freundii*)	98.32%	92.26%	90.46%	90.49%	90.99%	92.30%	92.52%

ANI was calculated by BLAST (referred as ANIb in JSpecies).

[a] This isolate, identified as *C. freundii* based on the phylogenetic tree (Fig. 4), was presented to show validity of the ANI analysis. The isolate has >95% identity against *C. freundii* CAV1321 and <95% identity against *C. braakii* CIP104554, *C. werkmanii* CIP104555, *C. youngae* CIP 105016, and *C. pasteurii* CIP 55.13.

Strain	Country	Year	Speices	ST	CC	Carbapenemase	In number	ESBLs/plasmid AmpCs
Kp 05.03S	Philippines	2010	K. pneumoniae	2286	405	IMP-26	UD	CTX-M-15
SMART1137	Japan	2013	K. pneumoniae	14	14	IMP-6	1321	CTX-M-2
SMART1134	Japan	2013	K. pneumoniae	14	14	IMP-6	UD	CTX-M-2
SMART322	Philippines	2011	K. pneumoniae	14	14	IMP-26	UD	CTX-M-15
SMART325	Philippines	2011	K. pneumoniae	14	14	IMP-26	UD	CTX-M-15
SMART327	Philippines	2011	K. pneumoniae	14	14	IMP-26	1310	CTX-M-15
SMART323	Philippines	2011	K. pneumoniae	14	14	IMP-26	1310	CTX-M-15
SMART878	Philippines	2013	K. pneumoniae	14	14	IMP-26	UD	CTX-M-15
SMART319	Philippines	2011	K. pneumoniae	14	14	IMP-26	UD	CTX-M-15
SMART882	Philippines	2013	K. pneumoniae	2289	14	IMP-26	UD	CTX-M-15
SMART321	Philippines	2011	K. pneumoniae	14	14	IMP-26	1310	CTX-M-15
SMART326	Philippines	2011	K. pneumoniae	14	14	IMP-26	1310	CTX-M-15
Kp 3 IMP	Philippines	2009	K. pneumoniae	626	-	IMP-26	UD	-
Kp 2 IMP	Philippines	2009	K. pneumoniae	626	-	IMP-26	1310	-
SMART875	Philippines	2013	K. pneumoniae	20	17	IMP-26	1310	CTX-M-15
SMART651	Thailand	2012	K. pneumoniae	70	70	IMP-14	687	SHV-5, SHV-12
SMART1131	Japan	2013	K. pneumoniae	37	37	IMP-6	722	CTX-M-2
SMART1133	Japan	2013	K. pneumoniae	37	37	IMP-1	1312	CTX-M-2
SMART1135	Japan	2013	K. pneumoniae	37	37	IMP-1	1312	CTX-M-2
SMART1136	Japan	2013	K. pneumoniae	37	37	IMP-1	1311	CTX-M-2
Kp 4 IMP	Australia	2009	K. pneumoniae	478	1310	IMP-4	UD	CTX-M-15
Kp 5 IMP	Australia	2009	K. pneumoniae	478	1310	IMP-4	UD	CTX-M-15
SMART1259	Taiwan	2014	E. cloacae cluster III	78	78	IMP-8	73	CTX-M-3
SMART399	Taiwan	2011	E. cloacae cluster III	78	78	IMP-8	73	SHV-5, SHV-12
SMART271	Taiwan	2010	E. cloacae cluster III	78	78	IMP-8	73	SHV-12
SMART409	Spain	2011	E. hormaechei subsp. oharae	182	182	IMP-13	1319	-
SMART1260	Australia	2014	E. hormaechei subsp. oharae	108	108	IMP-4	UD	-
SMART1291	Australia	2014	E. hormaechei subsp. steigerwaltii	90	90	IMP-4	809	SHV-12
SMART398	Taiwan	2011	E. hormaechei subsp. steigerwaltii	204	234	IMP-8	UD	-
SMART1276	Thailand	2014	E. hormaechei subsp. steigerwaltii	93	93	IMP-14	1314	CTX-M-15
SMART1234	Australia	2014	E. hormaechei subsp. steigerwaltii	133	133	IMP-4	UD	SHV-12
SMART316	Australia	2011	Citrobacter sp.	99	-	IMP-4	809	-
SMART314	Australia	2011	Citrobacter sp.	97	-	IMP-4	809	-
SMART958	Brazil	2013	C. freundii	100	-	IMP-1	1312	OXA-142
SMART315	Australia	2011	C. freundii	98	-	IMP-4	809	CTX-M-9
E. coli 7 IMP	India	2008	E. coli	443	205	IMP-1	1313	CTX-M-15
SMART1132	Japan	2013	E. coli	131	131	IMP-6	722	CTX-M-2
SMART640	Thailand	2012	E. coli	131	131	IMP-14	687	CTX-M-27

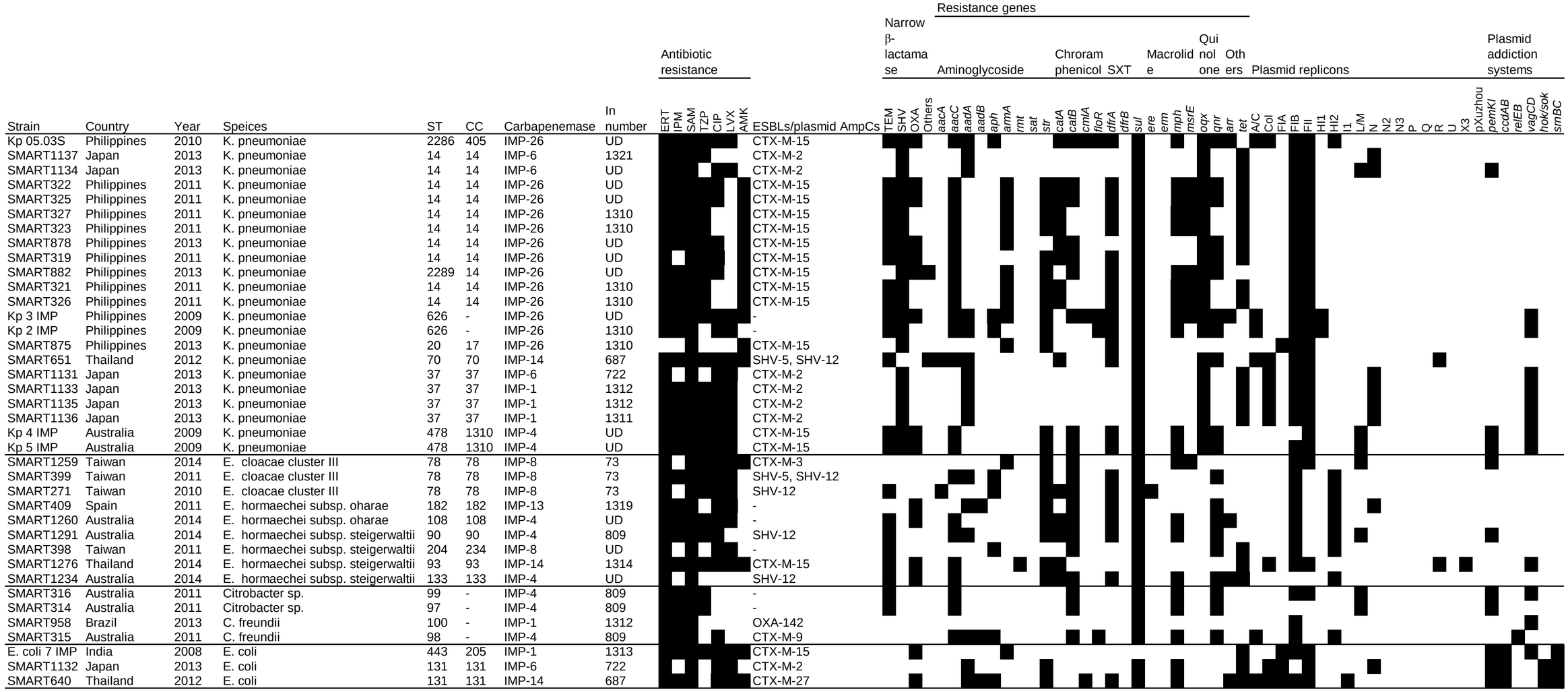

Fig. S1. Resistance genes, antibiotic susceptibilities, plasmid replicons, and plasmid addictions systems. The order of strains is the same as that in each phylogenetic tree (Figs. 2, 3, 4, S2). Abbreviations: ertapenem, ETP; imipenem, IPM; ampicillin/sulbactam, SAM; piperacillin/tazobactam, TZP; ciprofloxacin, CIP; levofloxacin, LVX; amikacin, AMK; trimethoprim/sulfamethoxazole, SXT. Non-susceptibility to antibiotics or the presence of genes were shown in black.

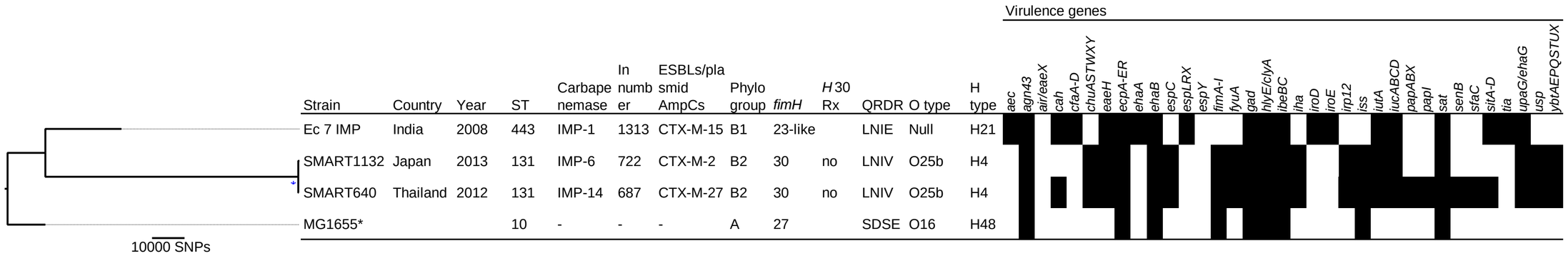

Fig. S2. Phylogenetic tree of IMP-producing *E. coli*. This maximum-likelihood phylogram is based on a 3,803,169 bp core genome and a total of 117,703 SNPs. The core genome was identified using E. coli MG1655 as a reference genome. The tree included 3 study isolates and one reference strains (marked with asterisk). The tree is rooted by using the outgroup of phylogenetic group A MG1655 and asterisks indicates bootstrap support > 90% from 100 replicates. QRDR (quinolone resistance-determining regions) genotypes are shown sequentially by the deduced amino acids of GyrA codons 83 and 87 and ParC codons 80 and 84.