

Title: High-level fluoroquinolone resistant *Salmonella enterica* serovar Kentucky ST198 epidemic clone with IncA/C conjugative plasmid carrying *bla*_{CTX-M-25} gene

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Abstract

Multidrug resistant *Salmonella* Kentucky strains have been isolated from turkeys in Poland since 2009. Multiple mutations within chromosomal genes *gyrA*, *parC* and *parE* were responsible for high-level ciprofloxacin resistance. One of the isolates was extended spectrum β -lactamase- (ESBL) positive: the strain 1643/10 carried a conjugative 167779 bps plasmid of IncA/C family. The sequence analysis revealed that it carried a *bla*_{CTX-M-25} gene and an integron with another β -lactamase encoding gene – *bla*_{OXA-21}. This is the first known report of a CTX-M-25 encoding gene both in Poland and in *Salmonella* Kentucky world-wide, as well as in the IncA/C plasmid. Analysis of the integron showed a novel arrangement of gene cassettes – *aacA4*, *aacC-A1* and *bla*_{OXA-21} where the latter might result from an intergeneric gene transfer. The study confirmed *Salmonella* Kentucky population isolated in Poland

belongs to global epidemics of high level fluoroquinolone resistant clone ST198 that can carry rare β -lactamase genes.

Keywords: Extended spectrum beta lactamases (ESBLs), plasmid-mediated resistance, quinolone resistance, IncA/C

Introduction

Resistance of *Salmonella* to critically important antimicrobials such as cephalosporins and fluoroquinolones is currently one of the major public health concerns. In usually self-limiting salmonellosis a scary scenario starts if the infection is invasive and caused by multi-drug resistant (MDR) strain harboring transmissible resistance and virulence determinants (Douard et al., 2010). *Salmonella enterica* subsp. *enterica* serovar (S.) Kentucky is known for decades with mostly antibiotic susceptible clones appearing in poultry in the US, Ireland (Boyle et al., 2010; Johnson et al., 2010; Melendez et al., 2010), and recently in exotic reptiles in Poland (Zajac et al., 2013). Currently the attention is focused on multilocus sequence type ST198 clone that emerged in Egypt and became MDR due to selective pressure of antimicrobial usage in poultry (Le Hello et al., 2011). International food production and supply as well as travels contributed to its rapid spread in Africa, Asia, Europe and North America (Beutlich et al., 2012; Bonalli et al., 2012; Le Hello et al., 2011; Mulvey et al., 2013; Turki et al., 2012) and is leading to global establishment of the challenging bacterial clone (Le Hello et al., 2013a). *Salmonella* control measures implemented in poultry are being hampered by various reservoirs of the pathogen including animals and food of animal origin, companion and wildlife animals, environment, and humans (Beutlich et al., 2012; Le Hello et al., 2013a; Zajac et al., 2013) as well as long-term environmental persistence due to biofilm formation (Turki et al., 2012). Furthermore, recent reports have pinpointed the possibility of human-borne clinical infection in animals (Guillon et al., 2013) and predilection of the clone to cause infection in turkey (Beutlich et al., 2012; Wasyl and Hoszowski, 2012).

Over the last decades multiple examples of MDR *Salmonella* serovars got attention mostly due to resistance encoded by *Salmonella* Genomic Island (SGI). The contribution of conjugal transfer of IncA/C helper plasmid pR55 for SGI acquisition has been recently shown (Douard et al., 2010). Continuous genomic rearrangements within SGI (Doublet et al., 2008) point out on the ongoing evolution of *S. Kentucky* ST198 epidemic clone (Beutlich et al., 2012; Le Hello et al., 2012; Mulvey et al., 2013; Wasyl and Hoszowski, 2012; Zając et al., 2013). The most notable features of a contemporary ST198 clone are MDR (amoxicillin, streptomycin, spectinomycin, gentamicin, sulfamethoxazole, and tetracycline) and heavy metals resistance related to *Salmonella* genomic island (SGI), high level fluoroquinolone resistance, and occasionally plasmid-mediated resistance to cephalosporins and carbapenems (Le Hello et al., 2013b; Le Hello et al., 2011; Le Hello et al., 2012; Turki et al., 2012). Typically for *Salmonella*, quinolone resistance results from spontaneous, consecutive mutations of chromosomal topoisomerases genes (*gyrA*, *gyrB*, *parC*, *parE*) and step-wise amino-acid substitutions are needed for high-level resistance. Resistance phenotype might be further modulated with multiple plasmid-mediated quinolone resistance mechanisms (PMQR) (Gunell et al., 2009; Le Hello et al., 2011; Luo et al., 2011; Wasyl et al., 2014).

The genes conferring antibiotic resistance located on plasmids with replicons belonging to IncI1, IncN, IncFIB, IncFIIA, IncW, IncA/C and IncL/M incompatibility groups were detected in *S. Kentucky*. Plasmids carried PMQR, β -lactam, cephalosporin, carbapenem, tetracycline, and azithromycin resistance genes (Garcia-Fernandez et al., 2009; Harrois et al., 2013; Le Hello et al., 2013b; Melendez et al., 2010). Moreover, ColV plasmids with IncFIB/FIIA replicons, encoding several virulence factors were responsible for enhanced colonization and virulence in poultry and *S. Kentucky* persistence in farm environment (Johnson et al., 2010). Mentioned above IncA/C plasmids constitute another large plasmid family found in *S. Kentucky* and broad range of hosts. The prototypic pRA1 plasmid was

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isolated in 1971 in Japan from tetracycline and sulfonamide resistant *Aeromonas hydrophila* and up-to date members of the family have been isolated worldwide from different human and animal pathogens (see del Castillo et al., 2013 and citations therein). The plasmid conserved backbone (74-100% sequence identity of IncA/C family members) comprises of a replication region, a stability module and a transfer moiety which displays sequence identity to integrative conjugative element (ICE) SXT/R391 (Johnson and Lang, 2012). The integration of various transposons (Tn) or insertion sequences (IS) introducing different resistance gene arrays into hotspots in plasmid sequences resulted in the particular diversity of IncC/A family (Johnson and Lang, 2012). In *S. Kentucky* IncA/C plasmids have been found carriers of few cephalosporin resistance genes (Le Hello et al., 2013a; Le Hello et al., 2013b) but up to our knowledge none of them coded for extended-spectrum β -lactamase (ESBL).

MDR *S. Kentucky* has emerged in Poland in turkey flocks and turkey meat in 2009 (Wasył and Hoszowski, 2012). Further studies confirmed ST198 being responsible for the epidemics (Le Hello et al., 2013a; Zajac et al., 2013). Current follow-up study aimed at identification of mechanisms responsible for high-level fluoroquinolone and cephalosporin resistance including detail characterization of plasmid carrying ESBL determinants.

Materials and Methods

Bacterial strains

Twenty-seven *S. Kentucky* isolates of previously described epidemiological relations representing the ST198 clone (Wasył and Hoszowski, 2012; Zajac et al., 2013) were further investigated. They were isolated between July 2009 and December 2010 from turkeys (N=15), turkey or unspecified poultry meat (N=5), food production hygiene checks (N=4), feed (N=2) and municipal sewage sludge (N=1). The following laboratory *Escherichia coli* recipient strains were used: for transformation TOP10 (F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*), ϕ 80*lacZAM15* *AlacX74* *nupG* *recA1* *araD139* Δ (*ara-leu*)7697 *galE15* *galK16* *rpsL*(Str^R)

endA1 λ) (Invitrogen, Life Technologies, CA, USA), in matings JE2571Rif^R (*leu thr thi lacY thy pil fla rif^R*) (Gołębiewski et al., 2007).

Antimicrobial resistance testing

Minimal inhibitory concentrations (MICs) of quinolones (nalidixic acid – Nal and ciprofloxacin – CIP) and cephalosporins (cefotaxime – CTX and ceftazidime – CAZ) were determined with microbroth dilution method (Sensititre®, Trek Diagnostic Systems, UK) and interpreted according to epidemiological cut-off values as described previously (Wasył and Hoszowski, 2012).

Identification of resistance determinants

Quinolone Resistance Determining Regions (QRDR) of the genes encoding for gyrase subunits A and B (*gyrA*, *gyrB*) and topoisomerase IV subunits C and E (*parC*, *parE*) and Plasmid Mediated Quinolone Resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6')-Ib-cr*) were tested as described previously (Wasył et al., 2014). Relevant QRDR amplicons were sequenced (Genetic Analyser 3500, Life Technologies). The nucleotide sequences were analyzed with SeqMan Pro (DNA Star Lasergene) and MEGA5 software (Center for Evolutionary Medicine and Informatics), compared against reference sequences and deposited in GenBank under Accession Numbers: KC172109 ÷ KC172135 (*gyrA*), KC172136 ÷ KC172162 (*parC*), and KC172163 ÷ KC172189 (*parE*). Identification of cephalosporin resistance determinant (PCR targeting *bla*_{CTX-M}) has been done as described previously (Wasył and Hoszowski, 2012) with final identification of the gene during plasmid sequence analysis.

Plasmid analyses

Previously described (Hoszowski et al., 1999) alkaline lysis method was used for plasmid profile screening with *E. coli* 39R and V517 as reference markers. Detailed plasmid characterization of four selected isolates was done with endonuclease S1-PFGE (Pulsed-field

gel electrophoresis; 1% SeaKem GTG agarose (FMC Bioproducts, USA; 6V/cm, switch time 1-25 s, 120°, 14°C, 20 h) (Barton et al., 1995) and PCR-based replicon typing (PBRT) using genomic DNAs (Carattoli et al., 2005). Electroporation was performed with One Shot® *TOP10* Electrocomp™ *E. coli* according to manufacturer's protocol (Invitrogen, USA).

Komentarz [i3]: Wyrzyciłam nawias, żeby nie było podwójnego

Plasmid conjugal transfer

One millilitre volumes containing 10⁹ CFU of donor and recipient strain LB cultures (Luria-Bertani broth; Biocorp, Poland) were mixed and incubated for 30 min at 37°C. Conjugation was stopped by vigorous vortexing for 30 s and placing the mating mixture on ice. Transconjugants were selected on LB agar (Biocorp) supplemented with cefotaxime (2 mg/L; Polfa Tarchomin, Poland) and rifampin (100 mg/L; Polfa Tarchomin). Solid mating was performed likewise, with an additional step of filtering the mating mixture through the sterile Millipore HA 45 µm filter (Millipore, USA) followed by incubation on LB plate. In the control experiments, the frequency of spontaneous mutations of both the donor and the recipient to the phenotype of transconjugants was assessed.

Plasmid DNA analysis

Transconjugant plasmid DNA was purified using the Plasmid Midi AX DNA kit 20 (A&A Biotechnology, Poland) and bacterial genomic DNA was purified using Genomic Mini kit (A&A Biotechnology). Pyrosequencing was performed on Genome Sequencer GS FLX+ Titanium (454) and the sequence was initially assembled using Newbler de Novo Assembler ver. 2.8 (454 Life Sciences, a Roche Company, USA). The gaps were filled after sequencing of PCR products generated from primers designed to the contig ends and finally the plasmid circle was closed and verified by restriction analysis performed *in vitro* and *in silico*. Plasmid sequence was analyzed using Clone Manager 9 Professional Edition ver. 9.2 and National Center of Biotechnology Basic Local Alignment Search Tool (BLAST). The whole p1643_10 sequence was compared and aligned to IncA/C plasmids using the Artemis Comparison Tool

(<http://sanger.ac.uk>) and mosaic resistance region was analyzed with ISFinder (<https://www-is.biotoul.fr/>) (Siguier et al., 2006). The complete nucleotide sequence of p1643_10 has been submitted to the GenBank database under Acc. No. KF056330.

Results

MICs

High level fluoroquinolone resistance was observed in 25 isolates ($MIC_{Nal} > 64$ mg/L and $MIC_{Cip} \geq 8$ mg/L). Isolate 1643/2010 and its transformants showed resistance to cefotaxime ($MIC_{CTX} > 4$ mg/L) and ceftazidime ($MIC_{CAZ} > 16$ mg/L), while the remaining isolates were susceptible to CTX and CAZ.

Komentarz [i4]: dodalam

Quinolone and cephalosporin resistance determinants

Sequencing of expected molecular weight amplicons of topoisomerases genes revealed substitutions in subunit GyrA (Ser83→Phe, Asp87→Tyr), ParC (Tyr57 →Ser, Ser80 →Ile) while no quinolone relevant mutations were found in *gyrB* and *parE* genes. The isolates were found negative for all tested PMQR determinants (data not shown). Isolate 1643/2010 and its transformants showed *bla*_{CTX-M} specific amplicon further identified *via* plasmid sequencing as *bla*_{CTX-M-25}.

Plasmid analysis

Numerous profiles comprising up to several plasmids of various molecular weight were noted in most of tested isolates (data not shown). High molecular weight plasmids were noted only in four isolates. S1-PFGE identified ca. 70-80 kb plasmids in three of them (1424/2010, 1719/2010, and 2238/2010), CTX-M producing isolate 1643/2010 carried plasmid of approximately 150 kb in size. The PBRT performed on genomic DNA classified plasmids of the three mentioned isolates to IncII group while the one observed in 1643/2010 – to IncA/C group. The plasmid of strain 1643/2010, p1643_10, was transferred by electroporation to laboratory TOP10 *E. coli* strain. Then, it was conjugally transferred from

the TOP10_{p1643_10} cells to JE2571Rif^R strain with the efficiency of $10^{-3} \div 10^{-4}$ per donor cell. Sequencing of the p1643_10 plasmid revealed that it was 167779 bps in size (FIG. 1), coded for 168 ORFs of which all had homologs in public databases. The plasmid shared extended homology of its backbone (replication-, stability-, conjugative transfer-encoding regions) with other sequenced IncA/C plasmids such as pR55, pTC2, pP91278 and others (FIG. 2). The majority of p1643_10 sequence (pos. 1 to 106549 and 147283 to 167779) displayed synteny (with at least 99% identity) with a large fragment of pTC2 (pos. 156977 to 29205). The remaining part of the sequence (pos.106550-147282) constitute a single mosaic MDR region of interesting structure (FIG. 3). All but two phage-related, of nineteen full or truncated transposase or integrase genes present in p1643_10 sequence had been found there. Moreover, this region comprises eleven genes involved in resistance to antimicrobials: *aphA1*, *strA*, *strB*, *sul2*, *aadB*, an integron with the novel array of gene cassettes: *aacA4*, *aacC-A1*, *bla_{OXA-21}* and *qacEA1* and *sull* in its 3'-CS, as well as the *bla_{CTX-M-25}* gene and the mercury resistance operon, *merEDACPTR*. A composite transposon with *aphA1* gene flanked by IS26 sequences (pos. 110625 to 113201) is also in 99% identical to part of pTC2, however, it is separated from the mentioned pTC2 fragment by the IS4321R integration site (pos. 106459 to 107789) downstream the *rhsA* gene. Such a genetic structure with the composite transposon situated next to the region comprising *strA*, *strB*, *sul2* genes and a truncated IncQ replicon, is identical to the region found in *S. Typhimurium* F-like plasmid pU302L (Chen et al., 2007). The other part of the p1643_10 sequence, from *tniA* gene, comprising the *mer* operon, to the plasmid end (pos. 134525 to 167779) shares 99% identity with pTC2 except of the site of ISEcp1 integration. This transposition brought *bla_{CTX-M-25}* gene, however, the ISEcp1 transposase gene was subsequently disrupted by IS50A insertion. Such *bla_{CTX-M-25}* genetic environment had been found in ca. 111 kb plasmid pCTX25 in Canada (Munday et al., 2004) (FIG. 3). The middle part of the mosaic MDR region is a hot spot of recombination and comprise the ISs

(ISc35, IS5D, ISEhe3 with *orfB* truncated), together with a second, single copy of the integron *intI1* gene, a putative resolvase gene and a region containing the short remnants group II intron and ISCR20. Only IS5D was found to have the complete terminal inverted repeats (IR) indicating probably the newest integration event. Interestingly, the large plasmid region outside the mosaic MDR region (from pos. ca. 107000 to 146000) was noticed to contain significantly less restriction enzymes recognition sites (for restrictases recognizing up to 10 sites) than the rest of the plasmid and overlapped with the plasmid backbone.

Discussion

Within the current study, resistance mechanisms to antimicrobials critical for public health: fluoroquinolones and cephalosporins have been identified in a selection of *S. Kentucky* isolated in Poland. Until the described *S. Kentucky* epidemics (Wasył and Hoszowski, 2012) none of *Salmonella* isolates monitored for antimicrobial resistance was ESBL positive. Clinical fluoroquinolone resistance (MIC > 1 mg/L) was observed in 0.8% of 2680 tested isolates. High-level MIC (MIC \geq 8 mg/L) was noted only in *S. Newport* and *S. Enteritidis* (two isolates each) but mostly in *S. Kentucky* (Wasył et al., 2014). Those isolates belonged to ST198 clone spreading in animals, foods, and humans in numerous countries (Beutlich et al., 2012; Bonalli et al., 2012; Le Hello et al., 2013a; Zając et al., 2013), while the susceptible ones represented reptile-related sequence type ST314 occurring in Poland (Zając et al., 2013). SGI1 variants harboring resistance gene clusters have been found responsible for diverse MDR phenotypes observed in *S. Kentucky* (Beutlich et al., 2012; Doublet et al., 2008; Le Hello et al., 2012). The presence of integrative and mobilizable SGI1 along with currently described chromosomal and transferable elements is of major scientific and epidemiological concerns (Le Hello et al., 2013a).

The genetic background of fluoroquinolone resistance confirmed that it is a step-wise phenomenon, affecting both *gyrA* and *parC* genes as targets to reach high-level ciprofloxacin

resistance (Le Hello et al., 2011). Most of the studied isolates revealed already described substitutions (Gunell et al., 2009; Luo et al., 2011; Wasyl et al., 2014) but the combination of Ser83→Phe and Asp87→Tyr in GyrA subunit and Ser80→Ile in ParC is extremely rare and, up to our knowledge, limited to epidemic population of *S. Kentucky* ST198 (Beutlich et al., 2012; Le Hello et al., 2013a; Le Hello et al., 2011). . Although *S. Kentucky* was reported to carry *qnrD* (Cavaco et al., 2009) and *qnrSI* (Garcia-Fernandez et al., 2009) none of the tested PMQR mechanisms was observed in the studied population. Our previous report of ST198 occurrence (Zajac et al., 2013) along with currently described multiple mutation pattern clearly confirmed Poland as a part of world-wide epidemics of MDR *S. Kentucky*.

Several cephalosporin resistance mechanisms, including extended-spectrum β -lactamases (SHV-12, CTX-M-1, CTX-M-15), cephalosporinases (CMY-2), and carbapenemases (OXA-48, VIM-2) (Boyle et al., 2010; Harrois et al., 2013; Le Hello et al., 2013b) have been described in *S. Kentucky*. Up to our knowledge this is not only the first study describing *bla*_{CTX-M-25} in *S. Kentucky*, but also the first report of this gene in IncA/C plasmid and its first appearance in Poland. CTX-M-25 have been firstly described in 2000 in *Escherichia coli* from Canadian patient (Munday et al., 2004). It is currently considered a representative for the subgroup of few β -lactamases rarely occurring worldwide (Vervoort et al., 2012). The unique genes of *bla*_{CTX-M} family that have been found so far in Poland code for CTX-M-3 and CTX-M-15 (Empel et al., 2008; Gołębiewski et al., 2007) in human clinical cases and CTX-M-1 in non-diseased animals (Wasyl et al., 2012). The *bla*_{CTX-M-25} carrying plasmid, p1643_10, was identified as member of a large IncA/C family (FIG. 2) composed of predominantly conjugative, broad-host range plasmids (Fricke et al., 2009; Johnson and Lang, 2012; Lindsey et al., 2009). The highly conserved backbone of these plasmids contains integration hot spots where different MDR regions are introduced to the sequence. In the p1643_10 sequence a single mosaic MDR region spanning ca. 40 kb have been identified,

integrated next to the complete *rhsA* gene (FIG. 3). The molecular history of this region is difficult to establish, however, the presence of complete terminal IRs of IS5D suggests the most recent transposition events. Also IS4321R presumably recently come into p1643_10 as its integration site distinguishes p1643_10 from pTC2 and pKEC-a3c plasmids. The *bla*_{CTX-M-25} way into p1643_10 after being mobilized by *ISEcp1* is hard to retrace since the pCTX25 sequence available in public databases is short. The class 1 integron detected in p1643_10 sequence contains a novel cassettes array. Plasmid pKEC-a3c from *Citrobacter freundii* recently published in GenBank contains an integron similar to that of p1643_10 however it lacks *bla*_{OXA-21} cassette. In p1643_10 integron containing two of gene cassettes, namely, *aacA4*, *aacC-A1*, together with *aadB* and *aphA1* genes confers resistance to entire spectrum of clinically used aminoglycosides. The other gene cassette, *bla*_{OXA-21}, is identical to the variant found in integrons of *Acinetobacter* and *Klebsiella* (Doublet et al., 2012; Yamamoto et al., 2011) and might be a result of a transgeneric gene transfer.

Epidemiological investigation in farm where the CTX-M-25 and OXA-21 producing isolate originated from did not show any other β -lactam resistant *Salmonella*, although multi-drug resistant *S. Kentucky* was isolated either from the same and the following turkey flocks (Wasył and Hoszowski, 2012). The plasmid carrying cephalosporin resistance genes must have been acquired from environmental source during turkey infection. This hypothesis might be supported by high IncA/C plasmids transferability in *S. Kentucky* (Fricke et al., 2009). The resistance probably did not offer any survival advantage to the recipient strain and it disappeared soon after. Such scenario cannot be excluded, although we failed to find any CTX-M positive commensal *Escherichia coli* in environmental samples taken few months after the incident of *bla*_{CTX-M-25} carrying *S. Kentucky*.

Finding of rare β -lactamase genes draw the attention to plasmids that can be easily acquired by *Salmonella* followed by further epidemic spread as described in OXA-48 and

VIM-1 producing *S. Kentucky* ST198 (Le Hello et al., 2013b). They also seem to be an important factor influencing genetic adaptation of *S. Kentucky*, both to increase its virulence (Johnson et al., 2010) and resistance to quinolones (Garcia-Fernandez et al., 2009) and cephalosporins (Bonalli et al., 2012). Multiple plasmids of broad-range molecular weight were also observed in our study (data not shown), but here we have focused on a single isolate carrying IncA/C plasmid. Considering genetic plasticity of *S. Kentucky* ST198, our results show that it might further alter its genome by acquisition of genetically diverse plasmids.

Based on current results as well as our previous and reviewed studies we claim that *S. Kentucky* population isolated in Poland belongs to world-wide epidemics of multidrug resistant and high level fluoroquinolone resistant *S. Kentucky*. Finding of rare cephalosporin resistance genes on a transferable plasmid multiplies possible consequences of human infection with a pathogen resistant to all antimicrobial classes used to treat systemic salmonellosis. Build on multiple studies showing genetic plasticity of *S. Kentucky* clone, current results indicate that the epidemic strain might further modify its gene content by acquisition of genetically diverse plasmids. Therefore *S. Kentucky* remains an intriguing challenge for future epidemiological studies and research.

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Komentarz [i5]: bez podkreślenia

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Conflict of interest statement

Nothing to declare.

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1 Figure captions

2 Fig. 1 Structure of the p1643_10 plasmid. Circular map of the p1643_10 plasmid. Genes are colour-
3 coded depending on functional annotations: plasmid replication (blue boxes), plasmid maintenance
4 (pink), DNA methylation (light pink), conjugative plasmid transfer (green),
5 transposition/recombination (yellow), resistance (red), others (light blue). The two external box-
6 circles depict coding strands; the third circle – pseudogenes. The inner black circle indicate
7 regions identical (min. 99%) with pTC2, another IncA/C plasmid (GenBank Acc. No. JQ824049).
8 The figure appears in colour in the online version of paper and in black and white in its print
9 version.

10 Fig. 2 Structure of the p1643_10 plasmid. Sequence comparisons of p1643_10 with IncA/C
11 plasmids: pTC2 (*Providentia stuartii*, Greece, 2008), pR55 (*Klebsiella pneumoniae*, France, 1969,
12 GenBank Acc. No. JQ010984), pP91278 (*Photobacterium damsela* subsp. *piscicida*, USA, 1991,
13 GenBank Acc. No. AB277724) and pKEC-a3c (*Citrobacter freundii* CFNIH1, USA, 2014,
14 GenBank Acc. No. CP007558). Comparisons were performed using the Artemis Comparison Tool
15 with a minimum score cut-off of 800 and a minimum percentage identity cut-off of 99%. Forward
16 and reverse matches are coloured in red and blue, respectively. The figure appears in colour in the
17 online version of paper and in black and white in its print version.

18 Fig 3. Sequence analysis of the mosaic resistance region of p1643_10. Arrows indicate ORFs
19 identified by sequence analysis: black filling – resistance genes, grey transposition associated, white
20 – of other function; truncated genes are marked as broken arrows. Tiny grey boxes indicate IS
21 terminal inverted repeats identified using ISFinder. Grey boxes beneath indicate parts of plasmids:
22 pU302L (Acc. No. AY333434.1), pCTX25 (partial sequence available - Acc. No. AF518567),
23 pKEC-a3c (Acc. No. CP007558), pTC2, with structures similar to p1643_10. All regions are drawn
24 to scale.

25