Turkish Journal of Molecular Biology & Biotechnology (2017) 2: 47-54

ORIGINAL ARTICLE

Turkish JMBB

pANKS2, a New Plasmid of *Salmonella typhimurium* and the Pathway of Plasmid Evolution for Antibiotic Resistance

Buse TÜREGÜN ATASOY[1], Mehmet TAŞPINAR[2], Devran GERÇEKER[1], A. Derya AYSEV[3], Birsel ERDEM[1], Fikret ŞAHİN[1]

[1]Department of Microbiology, Ankara University, School of Medicine, Ankara, Turkey

[2]Department of Medical Biology, Van Yüzüncü Yıl University, School of Medicine, Van, Turkey

[3]Department of Pediatrics, Ankara University, School of Medicine, Cebeci, Ankara, Turkey

Keywords

Plasmid, *Salmonella* Typhimurium, pAnkS2, transposons, antibiotic resistance

ABSTRACT • Information of antibiotic resistance is an important tool in epidemiologic control and treatment of infections. The mobile genetic elements such as plasmids and transposons responsible for developing resistance against mostly used antibiotics. Previously we isolated characterized and named a plasmid pAnkS from *Salmonella* Typhimurium (*S.* Typhimurium). Plasmid pAnkS is one of the few completely sequenced plasmids from S. Typhimurium and is made of transposition of Tn3 transposons of 4950 bp consisting of the left terminal repeat, Tn3-related *tnpR* and *tnpA* genes for transposition functions, ampicillin resistance bla_{TEM}, and the right terminal repeat into the p4821. In here another plasmid isolated from S. Typhimurium and named pAnkS2. The plasmid was analyzed partially for potential reading frames and structural features indicative of transposons and transposon relics. The overlapping restriction fragments of pAnkS2 were cloned into *E. coli* plasmid vectors, sequenced and analyzed with the BLAST programs. Plasmid pAnkS2 consist of EHEC plasmid p4821 as a core region and also complete Tn3 and Tn5-like transposons. pAnkS2 showed strong homology with plasmids described previously, pAnkS, and pNTP16 for sequences belong to p4821. P4821 is made the core region of pAnkS2 as in pAnkS, and pNTP16. pAnkS2 also carries Tn5-like transposon consisting of aminoglycoside 3'-phosphotransferase type II, bleomycin resistant genes and ORF sequence. Therefore, pAnkS2 was made up from the transpositions of the Tn5 transposon into the pAnkS which consist of transposition of Tn3 into the p4821. pAnkS2 shows multiresistant phenotype due to carrying aminoglycoside 3'-phosphotransferase type II, bleomycin and ampicillin sequences. The analysis of p4821, pNTP16, pAnkS and partial analysis of pAnkS2 revealed that pAnkS is a simple form of plasmid found in the salmonella and might be the precursor of the most of the salmonella plasmids carrying multiple resistance genes including pNTP16, and pAnkS2.

Correspondence: Fikret Şahin, Ankara University Medical Faculty, Microbiology Dept. Basic Science Buildings, 3rd floor Sihhiye-Ankara, Turkey 06100 • Tel: 011 90-312 492 1029 • E-mail: fsahin29@hotmail.com • Fax: 011 90- 312 310 6370

INTRODUCTION

Salmonellosis is an important public health problem throughout the world, resulting in hospitalizations and deaths (1). During the past decades, *Salmonella enterica* serovar Typhimurium infections have increased in many parts of the world (1). In general, the primary approach to the treatment and control of *Salmonella* infections is the use of antimicrobial agents. However, bacteria are becoming increasingly resistant to multiple antibiotics in many areas of the world (2). In a number of bacteria, resistance is acquired from mobile genetic elements including bacteriophages, transposons, and plasmids, and is subjected to frequent rearrangements (3). The presence of antibiotic resistance genes on transposable elements is a matter of concern with regard to the dissemination of the resistance genes via horizontal gene transfer (4). In particular, genes coding for resistances to ß-lactams or tetracyclines in Gram-negative bacteria have been associated with transposons. Genes coding for ß-lactamases of the TEM-type are among the most prevalent ß-lactam resistance genes in Gram-negative bacteria. The blaTEM-1 gene has been detected on transposon Tn3 which is also located either on conjugative and non-conjugative plasmids or on the chromosome. Tn3 and Tn1721 are members of the Tn3 family of transposons and exhibit similar mechanisms of replicative transposition (4). Moreover, both transposons have inverted terminal repeats of 35–38 bp and produce characteristic 5 bp direct repeats at their integration sites in the genome. This suggests that these extra resistance genes may be located in other chromosomal regions or on plasmids (4). Some of these resistance plasmids from various bacterial origins have been partially or completely sequenced (4,5).

We previously identified and characterized an ampicillin resistant gene carrying plasmid from clinically isolated and described as *Salmonella enterica* serovar Typhimurium that conferred the ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT) resistance phenotype. The plasmid we named as pAnkS is 8271 bp size. The complete analysis of pAnkS revealed that it was made of the transposition of the Tn3 transposon into the enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 plasmid p4821 which contain the basic requirements of a plasmid such as replication, stability and mobilization genes (6). Further analysis of pAnkS showed that it is precursor form of another S. Typhimurium plasmid pNTP16 which carries kanamycin resistance gene in addition to ampicillin gene (5). In this study, we isolated and described a new plasmid named pAnkS2 (plasmid Ankara *Salmonella* 2). pAnkS2 shows multiresistant phenotype due to carrying aminoglycoside 3'-phosphotransferase type II, bleomycin and ampicillin sequences and was made up from the transpositions of the Tn5 transposon carrying aminoglycoside 3'-phosphotransferase type II, and bleomycin into the pAnkS which consist of transposition of Tn3 into the p4821.

MATERIALS and METHODS

Bacteria, Plasmid and Culture Conditions

Plasmid pAnkS2 was found in a *Salmonella enterica subsp. enterica* serovar Typhimurium isolated from patient has gastroenteritis in Ankara. Plasmid DNA was prepared by alkaline lysis and subsequent purification. For preparation of the plasmid DNA bacteria were grown in L-broth (1% w/v NaCl, 1% w/v tryptone, 0.5% yeast extract, and pH7.5). For maintenance of recombinant plasmid, culture media supplemented with ampicillin to a final concentration of 100ug/ml.

Antibiotic Susceptibility Testing

After pAnkS2 transformed into the *E. coli* DH5α, antibiotic susceptibility testing was performed. The disk-diffusion assay was used to determine the antibiotic resistance profile of the strains using Mueller–Hinton agar (Difco Laboratories, Detroit, USA) following the National Committee for Clin-

ical Laboratory Standards (NCCLS) guidelines (NCCLS 2002). The antibiotic disks used were: ampicillin, ampicillin-sulbactam, carbenicillin, cephalothin, chloramphenicol, ciprofloxacin, ceftriaxone, cefuroxime, erythromycin, gentamicin, kanamycin, Polymyxin B, streptomycin, sulfisoxazole, tetracycline, trimethoprim, and trimethoprim-sulfamethoxazole. All antibiotic disks were purchased either from Difco or BBL (Becton–Dickinson, Sparks, MD).

General Recombinantion Techniques

After restriction mapping with enzymes, *BamH1, EcoRI, EcoRV HinfIII, HindIII, Not I, and XmnI*. *BamHI* fragments of pAnkS2 were cloned separately into pBSK (+) (Invitrogen, Groningen, The Netherlands) and transformed into *E. coli* DH5. Restriction endonuclease digestion was carried out according to the supplier's instructions (Fermentas- Life science technologies Lithuania). Purification of DNA fragments from agarose gels was performed with Gene-clean kit (GeneMark-Hopegen Biotechnology-China). Ligation and transformation experiments were carried out according to standard methods. Initial sequence analyses were conducted with the M13 reverse and forward primers for pUC/M13 vectors as described (7). For determination of the complete sequence, primer walking was carried out with oligonucleotide primers (IDT USA) designed from sequences previously obtained with the M13 reverse and forward primers.

Nucleotide Sequencing and Analysis of the Sequences

Nucleotide sequencing was performed with BigDye Terminator (PE Applied Biosystems) sequencing chemistry on an automatic DNA sequencer ABI 377 (Applied Biosystems) with universal and reverse primers for pBSK/M13 vectors and customized primer chosen during primer walking. Sequence analysis was carried out with the BLAST programs blastn and blastp (http://www.ncbi.nlm.nih.gov/BLAST/; last accessed 24 September 2016) as well as with the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html; last accessed 2016).

RESULTS

Restriction Enzyme Mapping

Restriction enzymes were tested for their ability to cleave plasmid pAnkS2. The enzymes *BamHI, EcoRI, EcoRV HinfIII, Not I,* and *XmnI* were tested. *BamHI* and *EcoRI* gave three bands individually and *EcoRV, HindIII* and *XmnI* gave two bands and NotI cut once the plasmid (Figure 1). Three *BamHI* fragments of pAnkS2 were cloned into the pBSK (+) cloning vector.

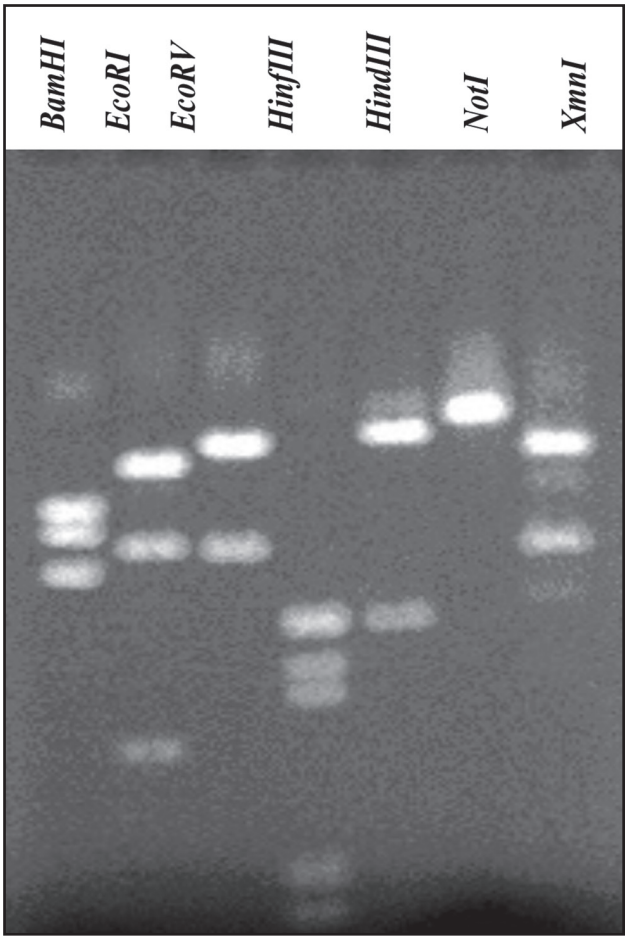

Figure 1 The restriction enzymes analysis of pAnkS2.

DNA Sequence

The nucleotide sequence of these fragment determined with an automized sequencer. For determination of the complete sequence, primer walking was carried out with oligonucleotide primers (IDT USA) designed from sequences previously obtained with the M13 reverse and forward primers.

Structure and Organization of Plasmid pAnkS2

In vitro susceptibility testing of *E. coli* DH5α: pAnkS2 transformants revealed that this plasmid mediates resistance to ampicillin, kanamycin, but susceptible to ampicillin-sulbactam, carbenicillin, cephalothin, chloramphenicol, ciprofloxacin, ceftriaxone, cefuroxime, erythromycin, polymyxin B, streptomycin, sulfisoxazole, tetracycline, trimethoprim, and trimethoprim-sulfamethoxazole. The partial nucleotide sequence analysis revealed that Plasmid pAnkS2 GC content is 48%. The determined nucleotide sequence analysis of pAnkS2 was used to search homology search with the BLAST programs. Analysis of pAnkS2`s core complex showed that this region harbored sequences essential for replication and stable maintenance of plasmid as also described by Haarmann et all (6) (Figures 2,3,5). Analysis of core complex revealed five open reading frames (Figures 2,3). Homology searches in the GenBank database library

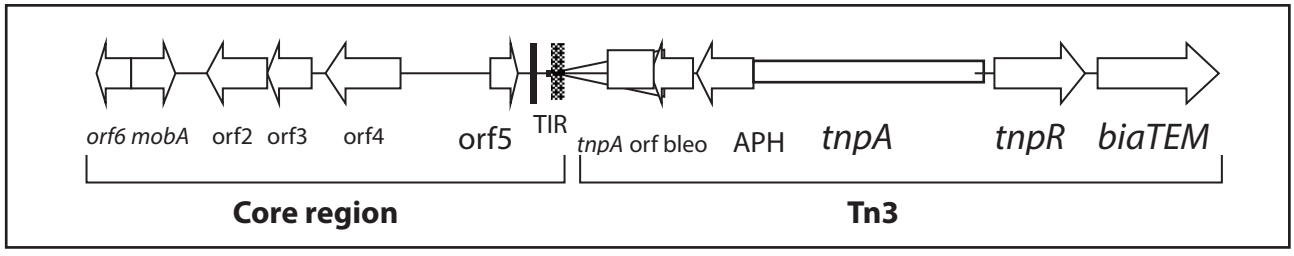

Figure 2 Linear map of plasmid pAnkS2. Relative size and orientation of open reading frames are marked by arrows and designated under the boxes.

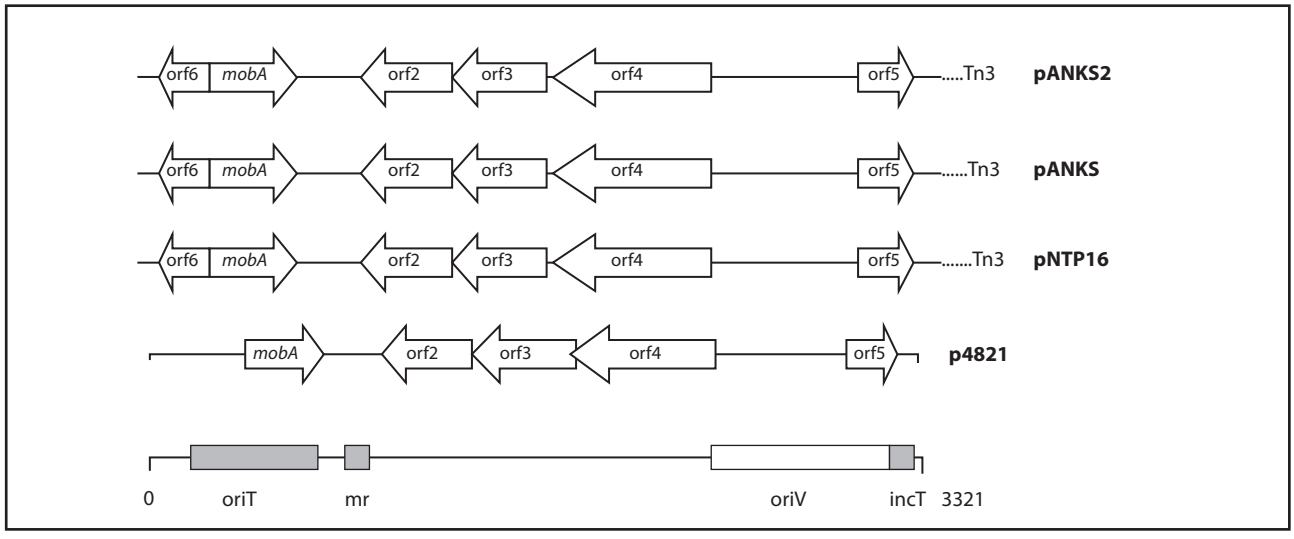

Figure 3 Linear map of plasmid p4821 and core regions of plasmids pAnkS2, pAnkS (accession no DQ916413), and pNTP16 (accession no L05392). Relative size and orientation of open reading frames are marked by arrows. The location of the orgin of transfer (oriT), the multimer resolution site (mr) and origin of replication (oriV) are depicted by boxes and designated inside the boxes.

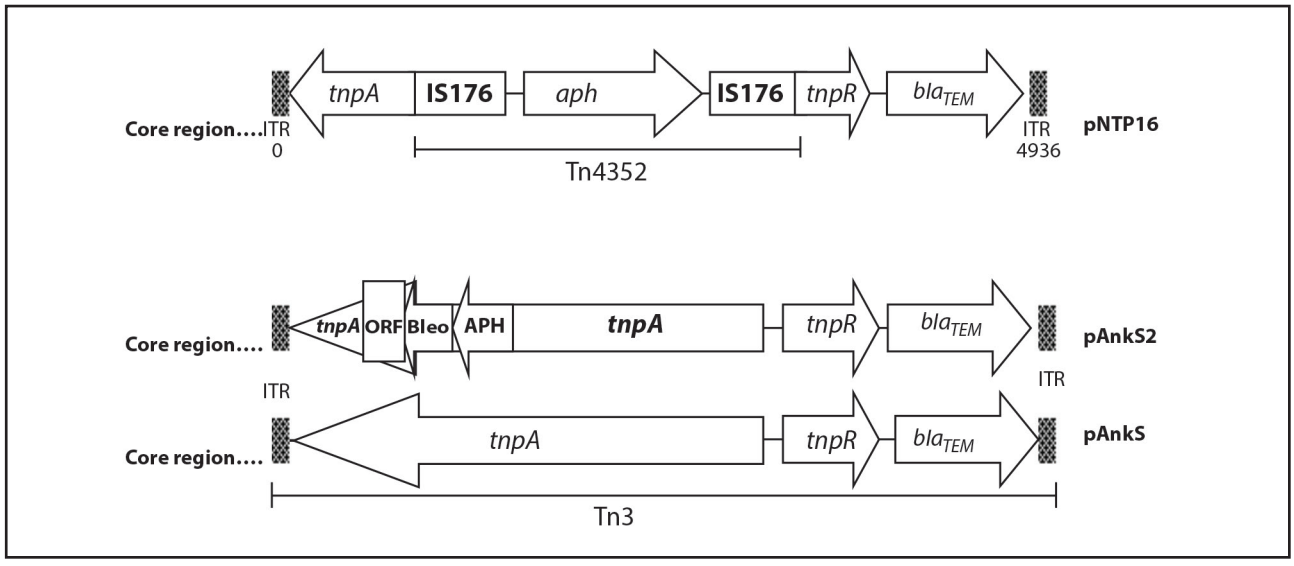

Figure 4 Comparative analysis of Tn3 (accession no. AJ634602), Tn3 flanking regions of pNTP16 pAnkS, pAnkS2. Tn5 containing aminoglycoside 3'-phosphotransferase type II and bleomycin resistance genes and ORF sequence was shown in the map of pAnkS2 and Tn4352 containing inverted IS176 segments and kanamycine resistance gene (aph) was shown in the map of pNTP16.

revealed close to 100% identity to the mobA gene and other ORFs of the S. Typhimurium plasmid pAnkS, NTP16 and of p4821. All functionally important features of cer are maintained in pAnkS2 and are also present in pAnkS, NTP16 and p4821. The sequence of pAnkS2 coordinates origin of transfer (oriT) is identical to the oriT of pAnkS, and pNTP16 and showed strong homology to the oriT of p4821 (Figure 3).

pAnkS2's core region is flanked by the Tn3 transposon (Figure 1) as in pAnkS. The sequenced Tn3 transposon part comprised the bla_{TEM} gene coding for a ß-lactamase, the genes tnpA and tnpR whose products are essential for transposition of Tn3 as well as the left and right terminal inverted repeat of Tn3. However, the partial analysis of the Tn3 transposons revealed that the Tn3 transposon of pAnkS2 separated by the tn5 transposon containing aminoglycoside 3'-phosphotransferase type II and bleomycin resistant genes and ORF sequence (Figure 4). The aminoglycoside 3'-phosphotransferase type II in pAnkS2 showed 100% identity to the complete sequence of the *Salmonella enterica*

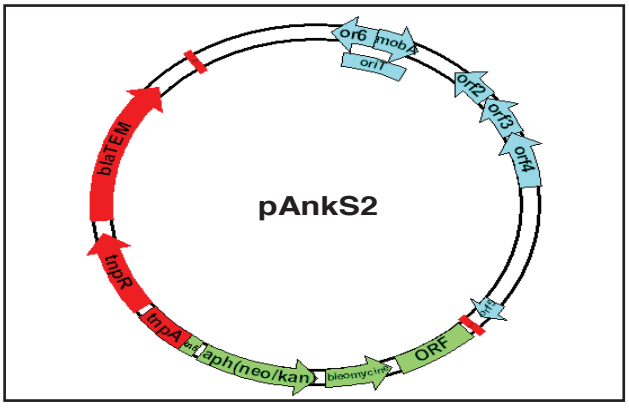

Figure 5 Circular map of plamid pAnkS2.

subsp. enterica serovar Dublin plasmid pMAK2 DNA (GeneBank-accession no: AB366441), and *Salmonella enterica subsp. enterica* serovar Indiana strain D90 plasmid pD90-3, (GeneBank-accession no: CP022453) and bleomycine was identical to the complete sequence of the pMAK2 DNA.

DISCUSSION

Plasmid pAnkS2 is a new *S.* Typhimurium plasmid contains Tn3 transposon contain parts of the

tn5 transposon carrying aminoglycoside 3'-phosphotransferase type II and bleomycin resistant genes and ORF sequence and plasmid p4821 isolated from the EHEC 0157:H7. Plasmid p4821 is a simple form of plasmid contains the only information necessary for its replication, stability, and mobilization (6). Analysis of plasmid p4821 patterns of EHEC strains showed that plasmid occurred at high frequencies (6). Presence of p4821 in the S. Typhimurium related plasmid pNTP1 and pNTP16, pAnkS and pAnkS2 suggest that p4821 is a phylogenetically long established plasmid; presumably distributed various enterobacteria and genetic transfer must have occurred among the bacteria.

P4821 is made the core region of pAnkS2 as in pAnkS and pNTP16. This core region was flanked by Tn3 in pAnkS2 as in pAnkS and pNTP16. Transposons of the Tn3 family do not show extended insertion site specificity, but appear to prefer A + T-rich sequences (4). When integrating into a new vector molecule, these transposons produce characteristic 5 bp direct repeats at the insertion site. The 5 bp direct repeats might be variable in different plasmids and genomes including TAAAA in pFPTB1 (GenBank accession no: AJ634602), TATTA in pLEW517 (GenBank accession no: DQ390454), TTATT in pINF5 (GenBank accession no: AM234722), TATAA in *Salmonella* Enteritidis gene (GenBank accession no: AB103092). In pAnkS the core complex and the transposon were separated on both sites by short direct repeats with the sequence TTCTT (7). This sequence also occurred in p4821; however, here it occurred only at one position and its obvious duplication on pAnkS at the boundaries to the Tn3 transposon led to suggest that it may function as a target site for the integration of transposon and segment might have integrated into the pAnkS basic replicon as one unit. When considering strong homology between pAnkS and p4821 and short direct repeats with the sequence TTCTT in both plasmid led to suggest that pAnkS is made of transposition of Tn3 into the p4821 plasmid at the TTCTT site (7).

As in pAnkS and pNTP16, pAnkS2 has the left and right inverted repeat of the Tn3 transposon. While pAnkS contain whole Tnp3 transposon, pNTP16 contain terminal 400 of 3000 of tnpA and terminal part of tnpR, ampR gene and inverted repeat regions in both sites. PNTP16 contain Tn4351 transposon carrying a protein identical to the aminoglycoside 3'-phosphotransferase of Tn903, in the missing portion of Tn3 (8).

Analysis of Tn4352 flanking sequences in Tn3 transposon of the pNTP16 provides evidence that pNTP16 is a derivative of pAnkS since they are strongly homologous to the terminal parts of tnpA and tnpR and ampR genes of Tn3 in addition to sharing the core region (Figure 6). Furthermore, it provides evidence that, subsequent to insertion within the resident Tn3 transposon, Tn4352 mediates an adjacent deletion of 2701 bps since there is no target duplication flanking the element. It is highly possible therefore that subsequent to the initial tn4352 transposition event into the pAnkS in either the tnpA or tnpR site a deletion has occurred, presumably promoted ISI171 and initiated at the one of the ends of Tn4352, resulting in the loss of 2701 bp of DNA (based on the Tn3 sequence) which contains most of sequence for tnpA and some of the sequence for tnpR (8).

Analysis of Tn5 flanking sequences in Tn3 transposon of the pAnkS2 provides evidence pAnkS2 contain parts of the tn5 transposon containing aminoglycoside 3'-phosphotransferase type II and bleomycin resistant genes and ORF sequences. In addition, they are strongly homologous to terminal parts of tnpA and tnpR and ampR genes of Tn3 in addition to sharing the core region. The analysis provides evidences that insertion sites of aminoglycoside 3'-phosphotransferase type II and bleomycin resistant genes and ORF sequences in Tn3 transposon are different than the insertion side of the Tn4352 in Tn3 transposon. While ISI76 divide the tnpA and tnpR in pNTP16, aminoglycoside 3'-phosphotransferase type II and bleomycin re-

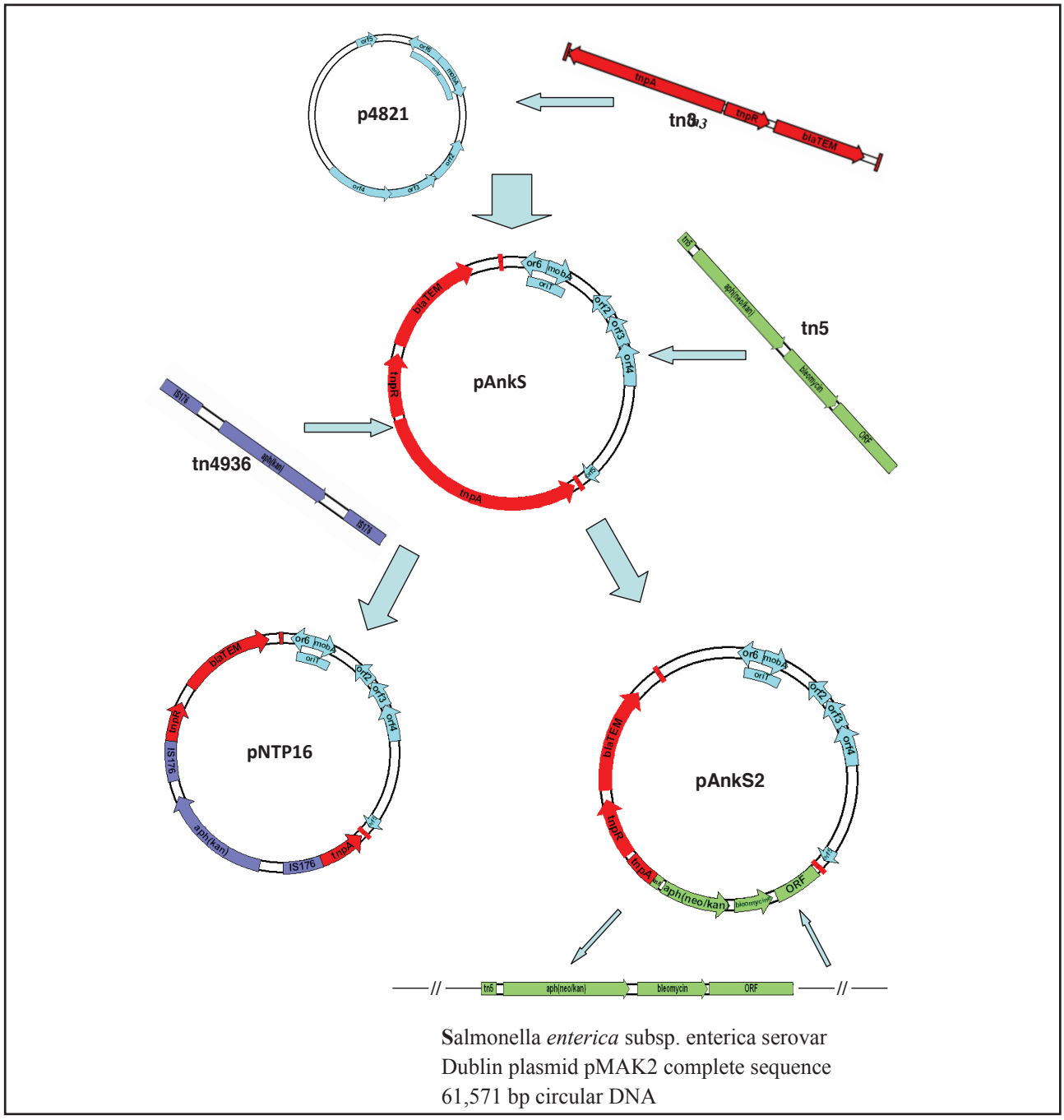

Figure 6 Diagram of the possible evolution of pAnkS, pAnksS2, pNTP16

sistant genes inserted in the tnpA side of the Tn3 Transposon in pAnkS2 (Figure 6). Previous studies had shown that the bacterial transposon Tn5 could insert into many sites in a gene (9). Since the sequence analysis is incomplate we couldn't determined the exact side of instertion of neomycin-kanamycin and bleomycin in Tn3 transposon. The blaTEM gene of pAnkS codes for 286 amino acid protein which identical the TEM extended-spectrum beta-lactamase (GenBank accession

no. DQ221256). The same as pAnkS2 comprised the blaTEM gene coding for a ß-lactamase.

Although p4821 plasmid originally found in England, the other Salmonella plasmids pNTP1, pNTP16, pAnkS, and pAnkS2 found in the close regions (Middle East countries) (10) and p4821 might be more universally available plasmid found in Enterobacteriaceae family. Previously, it was suggested that pNTP16 must be evolved from a plasmid containing only ampicillin resistance gene (5) (8). Therefore, pAnkS is the most likely plasmid which the precursor form of pNTP16, pAnkS2 and pMAK2 considering the evidences described above (Figure 6). We believe that description of pAnkS and pAnkS2 may help to understand the plasmid evolution better.

REFERENCES

1. MacFadden DR, Bogoch, II, Andrews JR: Advances in diagnosis, treatment, and prevention of invasive Salmonella infections. *Curr Opin Infect Dis* 2016, 29:453-458.
2. Quinn T, O'Mahony R, Baird AW, Drudy D, Whyte P, Fanning S: Multi-drug resistance in Salmonella enterica: efflux mechanisms and their relationships with the development of chromosomal resistance gene clusters. *Curr Drug Targets* 2006, 7:849-860.
3. McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F, et al.: Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. *Nature* 2001, 413:852-856.
4. Pasquali F, Kehrenberg C, Manfreda G, Schwarz S: Physical linkage of Tn3 and part of Tn1721 in a tetracycline and ampicillin resistance plasmid from Salmonella Typhimurium. *J Antimicrob Chemother* 2005, 55:562-565.
5. Cannon PM, Strike P: Complete nucleotide sequence and gene organization of plasmid NTP16. *Plasmid* 1992, 27:220-230.
6. Haarmann C, Karch H, Frosch M, Schmidt H: A 3.3-kb plasmid of enterohemorrhagic Escherichia coli O157:H7 is closely related to the core region of the Salmonella typhimurium antibiotic resistance plasmid NTP16. *Plasmid* 1998, 39:134-140.
7. Sahin F, Karasartova D, Gerceker D, Aysev AD, Erdem B: (A novel Salmonella Typhimurium plasmid, pAnkS: an example for plasmid evolution in antibiotic resistance). *Mikrobiyol Bul* 2008, 42:383-388.
8. Wrighton CJ, Strike P: A pathway for the evolution of the plasmid NTP16 involving the novel kanamycin resistance transposon Tn4352. *Plasmid* 1987, 17:37-45.
9. Berg DE, Schmandt MA, Lowe JB: Specificity of transposon Tn5 insertion. *Genetics* 1983, 105:813-828.
10. Lambert CM, Wrighton CJ, Strike P: Characterization of the drug resistance plasmid NTP16. *Plasmid* 1987, 17:26-36.