ST273 carbapenem-resistant Klebsiella pneumoniae carrying bla$_{NDM-1}$ and bla$_{IMP-4}$

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ST273 carbapenem-resistant *Klebsiella pneumoniae* carrying bla_{NDM-1} and bla_{IMP-4}

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Abstract

A carbapenem-resistant *Klebsiella pneumoniae* was recovered from human blood. Its whole genome sequence was obtained using both Illumina and long-read MinION sequencing. The strain belongs to ST273, which has been found recently and caused an outbreak in Southeast Asia. It has two carbapenemase genes $bla_{\text{NDM-1}}$ (carried by an ST7 IncN self-transmissible plasmid) and $bla_{\text{IMP-4}}$ (located on an self-transmissible IncHI5 plasmid). Non-KPC-producing ST237 may represent a lineage of carbapenem-resistant *K. pneumoniae*, which warrants further monitoring.

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Klebsiella pneumoniae is one of the most common pathogens of human infections and carbapenem-resistant *K. pneumoniae* (CRKP) has emerged as a major challenge to clinical management and public health globally (1). The production of carbapenem-hydrolyzing enzymes (carbapenemases) is the major mechanism mediating resistance to carbapenems in *K. pneumoniae*. There are a few types of carbapenemases and in *K. pneumoniae* the most common carbapenemase is KPC (a group of serine β-lactamases), followed by NDM and IMP (both of which are metallo-β-lactamases). The global dissemination of CRKP is largely mediated by the high-risk clonal complex 258 (CC258), which comprises ST11, ST258 and a number of closely related sequence types. However, other clones may also contribute to the international spread of CRKP. Recently, ST273 CRKP has been found in several countries (2-4), which warrants further investigations. We have identified a ST273 CRKP clinical strain carrying both bla_{NDM} and bla_{IMP} genes in our hospital and report its characterization here.

Strain WCHKP020034 was recovered from the blood of a 72-year-old male patient with pancreatitis at West China hospital. The strain was identified as *K. pneumoniae* by MALDI-TOF (Bruker, Billerica, MA) and Vitek II (bioMérieux, Marcy-l'Étoile, France). MICs of amikacin, aztreonam, aztreonam-avibactam, ceftazidime, ciprofloxacin, colistin, imipenem, meropenem, piperacillin-tazobactam, tigecycline and trimethoprim-sulfamethoxazole

against the isolate were determined using the broth microdilution method of the Clinical Laboratory Standards Institute (CLSI) (5). As there are no breakpoints of colistin and tigecycline from CLSI, those defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org/) were applied. The strain was resistant to aztreonam (MIC, 64 μg/ml), ceftazidime (>256 μg/ml), ciprofloxacin (256 μg/ml), imipenem (32 μg/ml), meropenem (64 μg/ml), piperacillin-tazobactam (>512/4 μg/ml) and trimethoprim-sulfamethoxazole (128/2,432 μg/ml), but is susceptible to amikacin (2 μg/ml), aztreonam-avibactam (0.25/4 μg/ml), colistin (1 μg/ml) and tigecycline (1 μg/ml). Acquired carbapenemase genes *bla*$_{GES}$, *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$ and *bla*$_{VIM}$ were screened as described previously (6-9) and the strain had *bla*$_{NDM}$ and *bla*$_{IMP}$. *bla*$_{NDM-1}$ and *bla*$_{IMP-4}$ were identified by amplifying and sequencing the complete coding sequence of *bla*$_{NDM}$ and *bla*$_{IMP}$.

The strain was subjected to whole genome sequencing with 150 × coverage using the HiSeq X10 Sequencer (Illumina, San Diego, CA), which generated 4,395,250 reads. Reads were trimmed using Trimmomatic (10) and were then assembled to 125 contigs (70 were ≥ 1,000 bp in length) with a 56.79% GC content using the SPAdes program (11). The *wzi* gene allele, which represents the capsular variation, of strain WCHKP020034 was 50, corresponding to several K types, i.e. K15, K17, K50, K51 and K52 with K15 being the best

74 match as predicted using Kaptive (12). None of the K types were K1, K2, and
75 K5, which are proposed as the hypervirulent members of *K. pneumoniae*. With
76 respect to virulence, strain WCHKP020034 had the *mrk* gene cluster
77 (*mrkA-B-C-D-F-H-I-J*), which encodes type 3 fimbrial expression (13) and is
78 seen in almost all *K. pneumoniae* isolates (1). Other known virulence genes
79 such as those encoding yersiniabactin, colibactin, allantoinase and aerobactin
80 were absent from strain WCHKP020034.
81
82 Strain WCHKP020034 belonged to sequence type 273 (ST273) as determined
83 using the *de novo* assembled genome sequence to query the multi-locus
84 sequence typing database of *K. pneumoniae*
85 (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). There were 10 additional
86 ST273 strains with whole genome sequence available in GenBank (accessed
87 by January 21, 2018; Table S1 in the Supplementary file). Genome sequences
88 of ST273 strains were retrieved from GenBank and were aligned with that of
89 strain WCHKP020034 using the Harvest Suite (14) with default settings. Single
90 nucleotide polymorphisms (SNPs) on recombination sites were removed by
91 Gubbins (15). The filtered SNPs were then used as input for inferring a
92 phylogenetic tree using RAxML (16) with the GTRGAMMA model and 1,000
93 bootstraps. Antimicrobial resistance genes in these genomes were identified
94 using ABRicate (https://github.com/tseemann/abricate) to query the ResFinder
95 database at the Center for Genomic Epidemiology

(http://genomicepidemiology.org/) and the *wzi* gene allele was predicted using Kaptive (12). Five strains carrying *bla*$_{NDM-7}$, a point mutant of *bla*$_{NDM-1}$, were recovered in 2013 in the Philippines and belong to a single cluster. No *wzi* allele was identified in these five strains. By contrast, strain WCHKP020034 was clustered with other ST273 strains (Figure 1) and was closest to strain COL-Kpn113 (carrying no *bla*$_{NDM}$, recovered in 2004 in Colombia) and strain K45-67 (carrying no *bla*$_{NDM}$ but *bla*$_{VIM-1}$, recovered in 2007 in Norway) with 116 to 123 SNPs difference respectively (Table S2 in the Supplementary file). Strains COL-Kpn113 and K45-67 had a *wzi* allele174, which is different from the allele 50 of strain WCHKP020034. The assembled genomes of ST273 strains were also typed using the cgMLST database (http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public&page=sequenceQuery) and a total of 951 genes were identified in all ST273 genomes. The 951 genes were identical in sequence among all ST273 strains other than WCHKP020034, while only 5 out of the 951 genes were different between strain WCHKP020034 and the other 10 ST273 strains (Table S3 in the Supplementary file). The relatively small number of SNPs and almost identical cgMLST results seen in strains from different geographic locations over such a long time frame suggests that ST273 might be highly clonal and merits further focused phylogenetic studies of this lineage. The *wzi* allele was different and even absent in ST273 strains but it is not uncommon to find more than one capsular types for strains of a single ST due to homologous

recombination of the capsular locus (17). Plasmids of the ST273 strains were predicted using PlasmidFinder but there is no plasmid replicon type present in all ST273 strains.

In addition to the two carbapenemase genes, strain WCHKP020034 had 24 intact antimicrobial resistance genes mediating resistance to aminoglycosides (*aac(3)-IId*, *ant(3'')-Ih-aac(6')-IId*, *aacA4*, *aadA1*, *aadA16, aph(3')-Ia*, *strA* and *strB*), β-lactams ($bla_{CTX-M-3}$ and $bla_{SHV-115}$), fosfomycin (*fosA*), macrolides (*mph(A)*), phenicol (*floR*), quinolones (*oqxA*, *oqxB*, *qnrB* and *qnrS1*), rifampicin (*arr3*), tetracycline (*tet(A)*), sulphonamides (*sul1* and *sul2*) and trimethoprim (*dfrA7*, *dfrA14* and *dfrA27*) (Table 1).

Conjugation experiments were preformed using filter- and broth-based methods at both 25 and 37 °C with the azide-resistant *Escherichia coli* strain J53 as the recipient. Transconjugants were screened using 1 μg/ml meropenem plus 150 μg/ml sodium azide and the presence of bla_{NDM-1} or bla_{IMP-4} in transconjugants was screened by PCR. bla_{NDM-1} and bla_{IMP-4} were carried on two self-transmissible plasmids, designated pNDM1_LL34 and pIMP4_LL34, respectively. To obtain the complete sequence of the plasmids, strain WCHKP020034 was subjected to sequencing using the long-read MinION Sequencer (Nanopore, Oxford, UK). The *de novo* hybrid assembly of both short Illumina reads and long MinION reads was performed using Unicycler (18) under the conservative mode for increased accuracy. Complete

circular contigs generated were then corrected using Plion (19) with Illumina reads for several rounds until no change was detected. Plasmid replicon type and plasmid multi-locus sequence type were determined using the PlasmidFinder and pMLST tools at http://genomicepidemiology.org/. The hybrid assembly of Illumina and MinION reads revealed that strain WCHKP020034 has a 5,295,791-bp circular chromosome and three large plasmids, i.e., the 58,953-bp pNDM1_LL34 of IncN (ST7), a 260,974-bp pIMP4_LL34 carrying bla_{IMP-4} and $bla_{CTX-M-3}$ with replicon types being unidentified by PlasmidFinder and a 130,688-bp plasmid carrying *qnrB* that contains an IncFII(K) and an IncQ1 replicon (designated pQnrB_LL34) (Table 1).

To understand the distribution of ST7 IncN plasmids, sequences of three alleles to define ST7 were concatenated and were then aligned against the nucleotide database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Plasmids of the ST7 IncN type were found in various species of the Enterobacteriaceae including *Citrobacter freundii*, *E. coli*, *Enterobacter cloacae*, *Enterobacter hormaechei*, *K. pneumoniae*, *Klebsiella oxytoca*, *Morganella morganii*, *Raoultella ornithinolytica* and *Raoultella planticola* from different countries, suggesting that ST7 IncN plasmids are widely distributed. In addition, ST7 IncN plasmids have been found to mediate the dissemination of bla_{IMP-4} in Enterobacteriaceae in different regions of China (20). Sequences

of all available ST7 IncN plasmids (n=32) were retrieved from the GenBank. Genes present on all ST7 IncN plasmids were considered as backbone genes, which were identified using OrthoFinder (21). Sequences of backbone genes were concatenated and were then aligned to infer a phylogenetic tree using RAxML (16) with a 1,000-bootstrap test. pNDM1_LL34 is clustered with several plasmids from various species (Figure 2). Among which, pNDM1_LL34 is closely related (99% coverage and 99% identity) to plasmid pNDM-BTR (GenBank accession number KF534788), which is also a ST7 IncN plasmid carrying bla_{NDM-1} that was recovered from an *E. coli* in Beijing, China, in 2013, as revealed by BLAST (blast.ncbi.nlm.nih.gov). The above findings suggest interspecies spread of a common IncN plasmid. On pNDM1_LL34 and pNDM-BTR, bla_{NDM-1}, several genes that are commonly associated with bla_{NDM-1}, and the quinolone-resistant gene *qnrS1* were bracketed by IS*26* (Figure 3). There were no 8-bp direct target repeats, which are the characteristic of the insertion of IS*26*, flanking the two copies of IS*26*, suggesting that homologous recombination contributed to the formation of such a structure. Nonetheless, two copies of IS*26* have the potential to form a composite transposon to mediate the mobilization of the intervening genetic components including bla_{NDM-1} and *qnrS1*. Outside of the two IS*26*, there was an interrupted Tn*3* family transposon, in which the transposase gene *tnpA* and both inverted repeats remain intact but the resolvase gene *tnpR* was truncated. The *fipA* gene that encodes a conjugal transfer inhibition protein and belongs

to the plasmid backbone was interrupted into two parts by the Tn*3* family transposon. The characteristic 5-bp direct target repeats flanked the Tn*3* family transposon, suggesting that the transposon inserted into *fipA*.

bla$_{IMP-4}$ was carried by a class I integron in the *bla*$_{IMP-4}$*-qacG2-aacA4* cassette array on pIMP4_LL34. Chloramphenicol resistance gene *catB3* is usually seen together with *bla*$_{IMP-4}$ in the *bla*$_{IMP-4}$*-qacG2-aacA4-catB3* cassette array but is absent from pIMP4_LL34. The integron is assigned In*1498* by INTEGRALL (http://integrall.bio.ua.pt/). By BLAST, the closest match of pIMP4_LL34 was p13190-VIM (88% coverage and 99% identity; GenBank accession no. MF344563) from *K. pneumoniae* in Beijing China. pIMP4_LL34 has a replicon, which has been proposed as IncHI5 (22) but has not been included into the database of PlasmidFinder. By BLAST using the 885-bp replication protein-encoding gene of the IncHI5 replicon, we identified 15 additional IncHI5 plasmids in GenBank. These plasmids were found in *K. pneumoniae*, *K. oxytoca*, *K. michiganensis*, *R. ornithinolytica*, and *R. planticola* and all but one were found at various locations in China. These findings suggest that IncHI5 plasmids have been circulated in China, which warrant further investigations. *bla*$_{CTX-M-3}$ was located downstream of IS*Ecp1* and upstream of a truncated orf477 gene. The IS*Ecp1-bla*$_{CTX-M-3}$-orf477 Δ unit was inserted in a gene encoding a protein of the Hok/Gef family with the presence of 5-bp direct target repeats, which is the characteristic of the transposition of IS*Ecp1*. It became

evident that IS*Ecp1* misrecognized a sequence in orf477, which has 8 out of 14 nucleotides matched with the right-hand inverted repeat (IRR), as it alternative IRR and then realized the mobilization of $bla_{CTX-M-3}$ into the Hok/Gef family protein-encoding gene.

In conclusion, we identified a ST273 CRKP carrying two carbapenemase genes bla_{NDM-1} and bla_{IMP-4}. bla_{NDM-1} was carried by an ST7 IncN self-transmissible plasmid and bla_{IMP-4} was located on an IncHI5 self-transmissible plasmid. This is yet another example of a clinical isolate containing multiple plasmids conferring resistance to carbapenems as we described before (23). The coexistence of plasmids may generate new platforms to mediate further spread of carbapenem-resistant genes and questions our knowledge of the extent to which plasmids conferring multi-drug resistance truly affect fitness of host bacteria. It creates a question as to why strains would possess multiple genes for the same resistance. The low diversity of ST273 isolates across continents and years suggests that the lineage merits further characterization.

Nucleotide sequence accession numbers. Complete sequences of the chromosome of strain WCHKP020034, pIMP4_LL23 and pNDM1_LL23 have been deposited into GenBank under the accession numbers CP025963, CP025964 and CP025965.

Acknowledgments

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323 *Enterobacter cloacae* producing both KPC and NDM carbapenemases:
324 characterization by whole genome sequencing. Antimicrob Agents
325 Chemother **59:**6625-6628.

326

327

328 Table 1. Antimicrobial resistance genes and their locations in strain

329 WCHKP020034.

	Size (bp)	Replicon type, pMLST	Antimicrobial resistance genes
Chromosome	5,295,791	-	*bla*$_{SHV-115}$, *fosA*, *oqxA*, *oqxB*
pNDM1_LL34	58,953	N (ST7)	*bla*$_{NDM-1}$, *dfrA14*, *qnrS*
pIMP4_LL34	260,974	IncHI5	*aacA4*, *bla*$_{CTX-M-3}$, *bla*$_{IMP-4}$, *sul1*
pQnrB_LL34	130,688	FII (K2:A-:B-), Q1	*aac(3)-IId*, *ant(3'')-Ih-aac(6')-IId*, *aadA1*, *aadA16, aph(3')-Ia, arr3*, *dfrA27*, *floR*, *mph(A)*, *qnrB*, *sul1*, *sul2*, *tet(A)*

330 ND, undetermined.

331

Figure legends

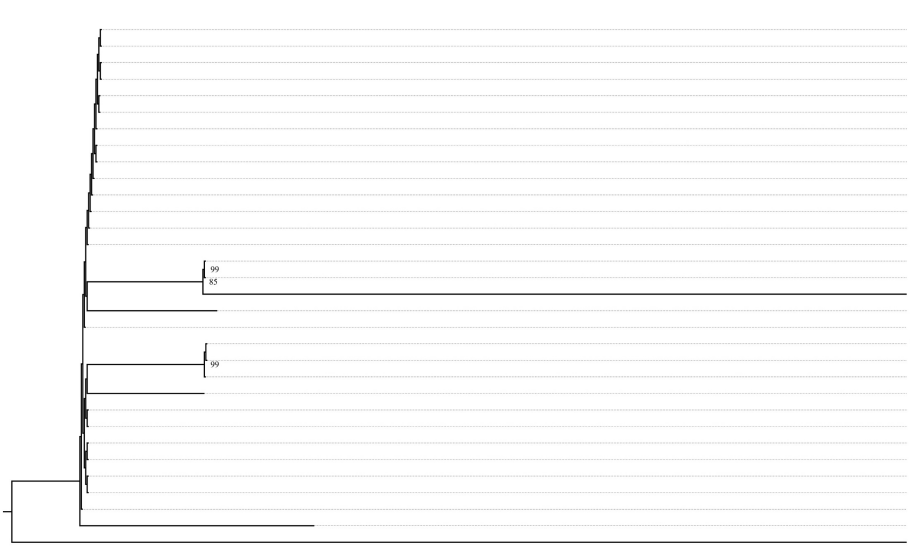
Figure 1. Maximum likelihood phylogenetic tree of *K. pneumoniae* ST273 strains with genome sequences available in the GenBank. The phylogeny is inferred from the recombination-filtered SNP alignment obtained by aligning either complete or draft genome of *K. pneumoniae* ST273 against the complete genome of WCHKP020034. The annotation denotes the presence of antimicrobial resistance genes as determined by Abricate.

Figure 2. Phylogenetic tree of ST7 IncN plasmids. The name, host species and accession numbers of the plasmids are shown. The tree was inferred using concatenated sequences of 26 genes belonging to the ST7 IncN backbone.

Figure 3. The genetic context of *bla*$_{NDM-1}$ on pNDM1_LL34. Genes between *bla*$_{NDM-1}$ and *qnrS1* are *ble* (mediating bleomycin resistance), *trpF* (encoding a phosphoribosylanthranilate isomerase), *dsbC* (encoding an oxidoreductase), *cutA1* (encoding an ion tolerant protein) and *groES*/*groEL* (encoding a chaperonin). The *tnpA* gene (encoding a transposase) and both inverted repeats (shown in blue poles) of a Tn*3* family transposon are outside the region flanked by IS*26*. *fipA* (encoding a conjugal transfer inhibition protein) is interrupted by the insertion of the Tn*3* family transposon with the characteristic the 5-bp direct target repeats (TATAT). Δ represents truncated genes or mobile

genetic elements.

Figure 1. Maximum likelihood phylogenetic tree of *K. pneumoniae* ST273 strains with genome sequences available in the GenBank. The phylogeny is inferred from the recombination-filtered SNP alignment obtained by aligning either complete or draft genome of *K. pneumoniae* ST273 against the complete genome of WCHKP020034. The annotation denotes the presence of antimicrobial resistance genes as determined by



Plasmids	Carbapenemases	Host	Accessions
pIMP-HK1500	bla_{IMP-4}	*Citrobacter freundii*	KT989599
pKOX105	bla_{VIM-1}	*Klebsiella oxytoca*	NC_014208
pIMP-FJ1503	bla_{IMP-4}	*Citrobacter freundii*	KU051710
pIMP-SH1506	bla_{IMP-4}	*Enterobacter cloacae*	KT989598
pECN580	bla_{KPC-2}	*Escherichia coli*	NC_025183
pIMP-SZ1515	bla_{IMP-4}	*Escherichia coli*	KT989376
pIMP-FS1505	bla_{IMP-4}	*Escherichia coli*	KT982615
pIMP-HZ1	bla_{IMP-4}	*Klebsiella pneumoniae*	KU886034
pCRKP-1-KPC	bla_{KPC-2}	*Klebsiella pneumoniae*	KX928750
pIMP-SZ1502	bla_{IMP-4}	*Escherichia coli*	KU051707
unnamed (FDAARGOS_430)	bla_{KPC-3}	*Klebsiella oxytoca*	CP023878
pK45-67VIM	bla_{VIM-1}	*Klebsiella pneumoniae*	NC_021622
pIMP-SZ1501	bla_{IMP-4}	*Klebsiella pneumoniae*	KU051708
pIMP-GZ1058	bla_{IMP-4}	*Escherichia coli*	KU051709
pIMP-KP1495	bla_{IMP-4}	*Klebsiella pneumoniae*	KU862632
pIMP-1495	bla_{IMP-4}	*Klebsiella pneumoniae*	KM977631
pCRKP-5-KPC	bla_{KPC-2}	*Klebsiella pneumoniae*	KX928751
p1220-IMP	bla_{IMP-4}	*Klebsiella pneumoniae*	KX711880
pIMP-HK1509	bla_{IMP-4}	*Escherichia coli*	KT982616
pNDM1_LL34	bla_{NDM-1}	*Klebsiella pneumoniae*	pNDM1_LL34
pNDM-BTR	bla_{NDM-1}	*Escherichia coli*	NC_022375
pNDM-CWH001	bla_{NDM-1}	*Citrobacter freundii*	NZ_CM008471
pIMP1496	bla_{IMP-4}	*Klebsiella pneumoniae*	KT982613
pIMP-DS1516	bla_{IMP-4}	*Escherichia coli*	KU726588
pIMP-GZ1517	bla_{IMP-4}	*Escherichia coli*	KT982618
pOW16C2	bla_{VIM-1}	*Klebsiella pneumoniae*	NC_025186
unnamed3	bla_{KPC-3}	*Raoultella ornithinolytica*	NZ_CP023894
pKo6	bla_{KPC-2}	*Klebsiella pneumoniae*	NC_025019
unnamed (FDAARGOS_429)	bla_{KPC-3}	*Raoultella planticola*	CP023875
p128379-IMP	bla_{IMP-4}	*Enterobacter hormaechei*	MF344559
pMR3-OXA181	$bla_{OXA-181}$	*Morganella morganii*	KM660724
p10677-IMP	bla_{IMP-4}	*Klebsiella pneumoniae*	MF344557

Figure 2. Phylogenetic tree of ST7 IncN plasmids. The name, host species and accession numbers of the plasmids are shown. The tree was inferred using concatenated sequences of 26 genes belonging to the ST7 IncN backbone.

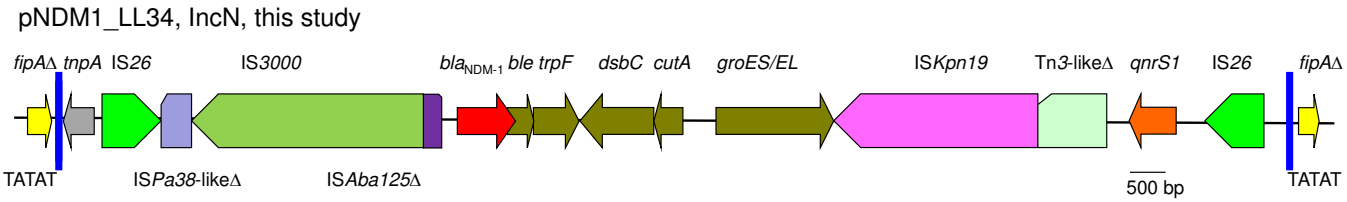


Figure 3. The genetic context of *bla*$_{NDM-1}$ on pNDM1_LL34. Genes between *bla*$_{NDM-1}$ and *qnrS1* are *ble* (mediating bleomycin resistance), *trpF* (encoding a phosphoribosylanthranilate isomerase), *dsbC* (encoding an oxidoreductase), *ctuA1* (encoding an ion tolerant protein) and *groES*/*groEL* (encoding a chaperonin). The *tnpA* gene (encoding a transposase) and both inverted repeats (shown in blue poles) of a Tn*3* family transposon are outside the region flanked by IS*26*. *fipA* (encoding a conjugal transfer inhibition protein) is interrupted by the insertion of the Tn*3* family transposon with the characteristic the 5-bp direct target repeats (TATAT). Δ represents truncated genes or mobile genetic elements.