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Genome Analysis of *Kingella kingae* Strain KWG1 Reveals How a β-Lactamase Gene Inserted in the Chromosome of This Species

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We describe the genome of a penicillinase-producing *Kingella kingae* strain (KWG1), the first to be isolated in continental Europe, whose bla_{TEM-1} gene was, for the first time in this species, found to be chromosomally inserted. The bla_{TEM} gene is located in an integrative and conjugative element (ICE) inserted in Met-tRNA and comprising genes that encode resistance to sulfonamides, streptomycin, and tetracycline. This ICE is homologous to resistance-conferring plasmids of *K. kingae* and other Gram-negative bacteria.

Kingella kingae is recognized as the first pathogen causing osteoarticular infections in children younger than 4 years of age (1–4). To date, penicillinase-producing strains harboring the bla_{TEM-1} gene on a plasmid have been isolated only in the United States, Iceland, and Israel (5). Recently, we described the first penicillinase-producing *K. kingae* strain to be found in continental Europe, KWG1, isolated from a child with arthritis (6). Using a Southern blot hybridization approach, we showed that the bla_{TEM-1} gene was chromosomally inserted in KWG1, in contrast to all of the penicillinase-producing strains previously described so far in the literature. Here, we report the complete genome sequence of KWG1.

Sequencing was performed by the Pacific Biosciences SMRT method using P4C2 chemistry. A total of 55,284 reads with a quality of ≥0.8 and a mean length of 4,456 bp were obtained, and a *de novo* assembly was performed by using the HGAP3 pipeline available through SMRT Analysis (version 2.2) from Pacific Biosciences. The mean depth of coverage was 91×. Polished assembly allowed us to obtain a single unique contig. Annotation was performed by Progenus.

Insertion sequence (IS) annotation was performed with ISfinder (http://www-is.biotoul.fr), and the new IS, IS*Kki1*, was deposited in its database (7). Conjugative system annotation was performed with CONJscan (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::CONJscan-T4SSscan) (8). Comparisons with other *Kingella* genomes and with antimicrobial resistance-associated plasmids were performed with the NCBI BLAST software (http://www.ncbi.nlm.nih.gov) and the Synteny Line Plot tool of the MaGe Platform (http://www.genoscope.cns.fr/agc/mage) (9).

The complete and circularized KWG1 genome is 2,140,065 bp long with a GC content of 46.48%. It contains 54 tRNAs, 4 rRNA operons, and 2,250 open reading frames (ORFs) coding for known or putative proteins.

Comparison with the *K. kingae* type strain ATCC 23330 genome (accession number AFHS00000000) shows that both strains have 1,842 orthologous ORFs in syntons (81.69%) and reveals that the bla_{TEM} gene encoding the *K. kingae* KWG1 β-lactamase is located on a large (74-kb) genomic island (ORFs 904 to 973) absent from the type strain and inserted in the vicinity of Met-tRNA. A putative phage integrase (ORF 2251) is present at its 3′ end (Fig. 1; Table 1). A second putative phage integrase is found in a 3-kb region (ORFs 967 to 973) located at the 5′ end. This region is composed of phage-like genes surrounded by two inverted copies of the same IS (IS*Kki1*) (Fig. 1; Table 1). The island has a global GC content of 49% and is composed of genes encoding resistance to various antimicrobial compounds, transposase genes, genes of phage origin, and genes associated with plasmid functions (transfer or replication). Among those encoding plasmid functions, CONJscan (8) identified 11 contiguous ORFs (955 to 965) as an MPF_T (mating pair formation proteins similar to the archetypal T-DNA conjugation system of *Agrobacterium tumefaciens* plasmid Ti) type IV secretion system involved in conjugative transfer (10); it also detected a coupling protein (TraG, ORF 946) and a relaxase (ORF 944). All of these colocalizing elements define a full conjugative system, and consequently, the genomic island can be considered an integrative and conjugative element (ICE) (11).

ICEs are mobile genetic elements found in about 18% of prokaryotic chromosomes and display both plasmid and phage features (8, 11). Like plasmids, they harbor a conjugative system composed of a type IV secretion system (MPF_T, ORFs 955 to 965), a coupling protein (ORF 946), and a relaxase (ORF 944). Like phages, they integrate into tRNA (tRNA-15) genes with integrases (ORF 2251). Evolutionary analyses suggest that ICEs derive from conjugative plasmids that acquired the phage-like ability to integrate into the chromosomes of bacteria (8). Of note, the KWG1 ICE harbors two putative plasmid replication genes (ORFs 924 and 927).

The presence of a 47-bp direct repeat (DR) sequence (RPT45590902; GAC TCA TAA TCC CTT GGT CGT GGG TTC GAA ACC CAC CCG ACC CAC CA) within the Met-tRNA gene

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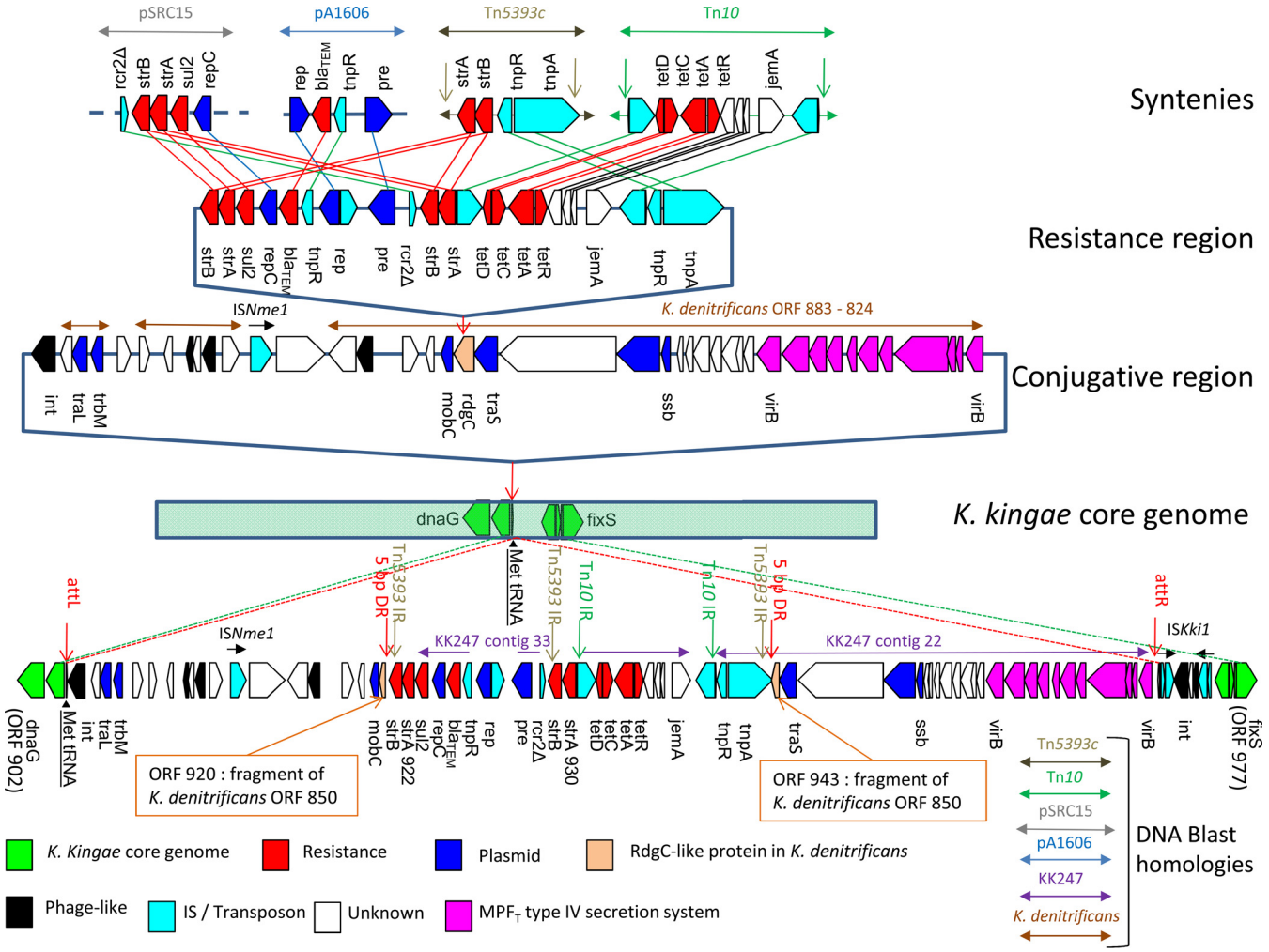

FIG 1 Schematic representation of the putative genetic events that led to chromosomal insertion of the bla_{TEM} gene in the *K. kingae* KWG1 genome and the genetic organization of the genomic region spanning bases 846108 to 926548. ORFs are represented by block arrows oriented according to the reading frame and color coded according to their putative functions or BLAST homologies. Green, *K. kingae* core genome (based on synteny with the *K. kingae* type strain ATCC 23330 genome); red, antimicrobial resistance; dark blue, plasmid transfer or replication; light salmon, RdgC-like protein in *K. denitrificans* strain ATCC 33394 (fragmented in KWG1); black, phage-like gene; light blue, IS or transposon (transposase, resolvase, and other); magenta, MPF_T type IV secretion system; white, unknown function without homology or synteny with the *K. kingae* type strain genome. Transposons Tn*10* and Tn*5393c*, as well as BLAST similarities to *Salmonella enterica* plasmid pSRC15, *H. influenzae* plasmid pA1606, *K. kingae* strain KK247, and *K. denitrificans* strain ATCC 33394, are indicated by colored horizontal double arrows. Syntenies with KWG1 are indicated by lines connecting ORFs with >50% identity on >80% of the shortest sequence. Isolated ISs (IS*Nme1* and IS*Kki1*) are indicated by black horizontal arrows. Putative right and left *att* sites (*attR* and *attL*, respectively) and the 5-bp DR generated by insertion of the resistance region in the RgdC-like ORF are indicated by vertical red arrows. Tn*5393c* IRs (Tn*5393* IR) and the 23-bp terminal IRs flanking Tn*10* (Tn*10* IR) are indicated by vertical green arrows.

and at the 5′ end of the MPF_T cluster could represent an *att* site for the insertion of bacteriophages and/or ICEs (Table 1; Fig. 1). Resistance genes for streptomycin (*strB* and *strA*), sulfonamides (*sul2*), penicillin (bla_{TEM}), and tetracycline (*tetR*, *tetA*, *tetC*, *tetD*) explaining the resistance profile of KWG1 are grouped together with genes encoding transposases and integrases in a small 25-kb region surrounded by two fragments of a truncated ORF encoding a putative exonuclease of the RdgC family (ORFs 920 and 943). Within this resistance region, streptomycin resistance-associated genes *strA* and *strB* are duplicated (ORFs 922 and 930 for *strA*, ORFs 921 and 2253 for *strB*). The resistance region is flanked by the inverted repeat (IR) of Tn*5393* and a 5-bp (ATAAT) DR, suggesting direct insertion into the RdgC family ORF (Fig. 1).

The 74-kb-long ICE is 97% similar to a region of the *Kingella denitrificans* ATCC 33394 genome (GenBank accession number NZ_GL870929). This region contains the same conjugative system and thus also corresponds to an ICE but notably lacks two features, the 25-kb region encoding antimicrobial resistance and the 3-kb region at the 5′ end corresponding to ORFs 967 to 973 (Fig. 1). In *K. denitrificans*, this ICE is inserted in the vicinity of Asn-tRNA, with bases 68111 to 69268 of contig 15 (GenBank accession number AEWV01000015) probably encoding an undetected phage-like integrase that is close to a *Kingella oralis* putative phage integrase (GenBank accession number EEP68425; 80% identity on 86% coverage) but clearly different from KWG1 ORF 2251 (52% identity on 92% coverage). Interestingly, in *K. denitrificans*, the putative exonuclease of the RdgC family (ORF 850) corresponding to fragmented ORFs 920 and 943 of KWG1 is com-

TABLE 1 Predicted ORFs, RNAs, and other genetic structures identified in the region of the *K. kingae* KWG1 genome spanning bases 846108 to 926548

Object	Gene	Product	Frame	Start	End	Length (bases)	GC content	Category
ORF-0902	*dnaG*	DNA primase	−2	846108	847871	1,764	0.4921	Core genome
ORF-0903		Conserved protein of unknown function	−2	847971	849143	1,173	0.5107	Core genome
tRNA-15		Met-tRNA	1	849282	849357	76		RNA
RPT45590902		DR (putative AttL)	1	849311	849357	47		DR
ORF-2251	*int*	Putative site-specific recombinase, phage integrase family	−2	849303	850427	1,125	0.5060	Phage-like
ORF-0904		Conserved protein of unknown function	−3	850916	851464	549	0.4699	
ORF-0905	*traL*	TraL protein	−2	851451	852194	744	0.5013	Plasmid
ORF-0906	*trbM*	TrbM	−1	852373	852915	543	0.5378	Plasmid
ORF-0907		Conserved membrane protein of unknown function	3	853617	854255	639	0.4444	
ORF-0908		Conserved protein of unknown function	1	854677	855135	459	0.4597	
ORF-0909		Conserved protein of unknown function	−3	855839	856228	390	0.4923	
ORF-0910		DNA-binding helix-turn-helix protein	−3	856895	857209	315	0.4984	Phage-like
ORF-0911		Conserved protein of unknown function	−2	857193	857573	381	0.4777	
ORF-0912		DNA-binding helix-turn-helix protein	−2	857673	858275	603	0.4046	Phage-like
ORF-0913		Conserved protein of unknown function	1	858604	859422	819	0.5678	
ORF-0914		Transposase, IS*5* family, IS*Nme1*	1	859948	860955	1,008	0.5179	Transposon
ORF-0915		Conserved exported protein of unknown function	1	861187	863475	2,289	0.5457	
ORF-0916		Conserved protein of unknown function	−3	863675	864952	1,278	0.4030	
ORF-0917		Phage protein Gp37/Gp68	−1	864991	865734	744	0.4382	Phage-like
ORF-0918		Conserved protein of unknown function	1	867187	867918	732	0.5601	
ORF-2252		Conserved protein of unknown function	−2	868137	868511	375	0.4880	
ORF-0919	*mobC*	Bacterial mobilization protein MobC	−3	869015	869545	531	0.4953	Plasmid
ORF-0920		Recombination-associated protein RdgC (fragment 1)	−1	869617	869997	381	0.5696	
		5-bp DR flanking resistance region	1	870124	870128	5		DR
misc_feature870129D		IR of Tn*5393*	1	870129	870208	80		Transposon
ORF-0921	*strB*	Streptomycin phosphotransferase B	−1	870235	871071	837	0.5591	Resistance
ORF-0922	*strA*	Streptomycin 3″-kinase	−2	871071	871874	804	0.5609	Resistance
ORF-0923	*sul2*	Dihydropteroate synthase type-2	−2	871935	872750	816	0.6078	Resistance
ORF-0924	*repC*	RepC	−2	873060	873869	810	0.6556	Plasmid
ORF-0925	bla_{TEM}	TEM-1 β-lactamase	−1	873985	874845	861	0.4925	Resistance
ORF-0926	*tnpR*	Tn*3*-like transposon resolvase, transposon Tn*2a*	−2	875028	875585	558	0.5269	Transposon
ORF-0927	*rep*	Replication protein	−2	875919	876848	930	0.3527	Plasmid
ORF-0928		Integrase core genome domain protein	1	876898	877704	807	0.5279	Transposon
ORF-0929	*pre*	Plasmid recombination enzyme	−2	878208	879479	1,272	0.3852	Plasmid
ORF-2255		Putative transposase zinc-binding domain, fragment of ISCR2	+3	880263	880544	282	0.5890	Transposon
misc_feature880548D		IR of Tn*5393*	1	880548	880627	80		Transposon
ORF-2253	*strB*	Streptomycin phosphotransferase B	−1	880654	881490	837	0.5591	Resistance
ORF-0930	*strA*	Streptomycin 3″-kinase	−2	881490	882293	804	0.5609	Resistance
		9-bp repeat of Tn*10* insertion	1	882338	882346	9		Transposon
misc_feature882340D		Terminal IR of transposon Tn*10*	1	882340	882362	23		Transposon
misc_RNA_3		RNA-OUT	−1	882385	882446	62		RNA
ORF-0931		Transposase, IS*4* family, IS*10*	3	882447	883655	1,209	0.4475	Transposon
ORF-0932	*tetD*	Transposon Tn*10* TetD protein	−2	883665	884081	417	0.3597	Resistance
ORF-0933	*tetC*	Transposon Tn*10* TetC protein	3	884094	884762	669	0.3259	Resistance
ORF-0934	*tetA*	Class B tetracycline resistance protein	−1	884875	886080	1,206	0.4328	Resistance
ORF-0935	*tetR*	Class B tetracycline repressor protein from transposon Tn*10*	1	886162	886737	576	0.4097	Resistance
ORF-0936	*jemC*	Conserved protein of unknown function	−1	886762	887385	624	0.4119	
ORF-0937		Conserved protein of unknown function	−3	887456	887842	387	0.3902	
ORF-0938	*jemB*	Conserved protein of unknown function	−2	887835	888134	300	0.3533	
ORF-0939	*jemA*	Glutamate transporter	2	888599	889804	1,206	0.3947	
ORF-0940		Transposase, IS*4* family, IS*10*	−1	890170	891378	1,209	0.4508	Transposon
misc_RNA_4		RNA-OUT	1	891379	891440	62		RNA
misc_feature891463R		Terminal IR of transposon Tn*10*	−1	891463	891485	23		Transposon
		9-bp repeat of Tn*10* insertion	−1	891479	891487	9		Transposon

(Continued on following page)

TABLE 1 (Continued)

Object	Gene	Product	Frame	Start	End	Length (bases)	GC content	Category
ORF-0941	*tnpR*	Putative resolvase for transposon Tn*5393*	−1	891514	892128	615	0.6065	Transposon
ORF-0942	*tnpA*	Transposase TnpA for transposon Tn*5393*	3	892254	895139	2,886	0.6195	Transposon
misc_feature895093R		IR of Tn*5393*	−1	895093	895172	80		Transposon
		5-bp DR flanking resistance region	1	895173	895177	5		DR
ORF-0943		Recombination-associated protein RdgC (fragment 2)	−2	895173	895565	393	0.5838	
ORF-0944	*traS*	TraS relaxase	−3	895562	896680	1,119	0.5621	Plasmid
ORF-0945		Toprim domain protein	−1	896779	902313	5,535	0.5429	
ORF-0946		TraG/TraD family protein	−3	902333	904372	2,040	0.5520	Plasmid
ORF-0947	*ssb*	Single-stranded DNA-binding protein 1	−2	904467	904880	414	0.5845	Plasmid
ORF-2254		Putative metalloprotease	+2	904925	905263	339	0.5580	
ORF-0948		Conserved protein of unknown function	−2	905223	905486	264	0.5568	
ORF-0949		Conserved protein of unknown function	−3	905618	905983	366	0.5519	
ORF-0950		Conserved protein of unknown function	−1	905980	906753	774	0.5581	
ORF-0951		Conserved protein of unknown function	−2	906798	907319	522	0.4540	
ORF-0952		Conserved protein of unknown function	−3	907316	908230	915	0.5607	
ORF-0953		Conserved membrane protein of unknown function	−1	907999	908325	327	0.5199	
ORF-0954		Conserved exported protein of unknown function	−1	908380	908844	465	0.4409	
ORF-0955	*virB11*	P-type DNA transfer ATPase VirB11	−1	909004	910104	1,101	0.5595	MPF_T
ORF-0956	*virB10*	Bacterial conjugation TrbI-like protein	−3	910190	911464	1,275	0.5137	MPF_T
ORF-0957	*virB9*	Putative P-type conjugative transfer protein VirB9	−1	911461	912300	840	0.5417	MPF_T
ORF-0958	*virB8*	VirB8 protein	−1	912313	913056	744	0.4449	MPF_T
ORF-0959	*virB7*	Conserved protein of unknown function	−2	913275	913754	480	0.4812	MPF_T
ORF-0960	*virB6*	TrbL/VirB6 plasmid conjugal transfer protein	−1	913786	914748	963	0.4798	MPF_T
ORF-0961	*virB5*	Type IV secretion system protein	−2	914808	915446	639	0.4507	MPF_T
ORF-0962	*virB4*	Type IV secretion/conjugal transfer ATPase, VirB4 family	−3	915548	918103	2,556	0.5082	MPF_T
ORF-0963	*virB3*	Type IV secretory pathway, VirB3-like protein	−2	918015	918443	429	0.4452	MPF_T
ORF-0964	*virB2*	Conserved membrane protein of unknown function	−3	918464	918784	321	0.5109	MPF_T
ORF-0965	*virB1*	Type IV secretion system protein VirB1	−3	918941	919747	807	0.5502	MPF_T
RPT45590902		DR (putative AttR)	1	919949	919995	47		DR
ORF-0966		Protein of unknown function	−1	920122	920250	129	0.4496	
ORF-0967		Transposase, IS*Kki1* ORF A	2	920342	920578	237	0.4093	Transposon
ORF-0968		Transposase, IS*Kki1* ORF B	2	920606	921172	567	0.3369	Transposon
ORF-0969	*int*	Putative site-specific recombinase, phage integrase family	−3	921179	922090	912	0.4748	Phage-like
ORF-0970		Conserved protein of unknown function	−3	922112	922399	288	0.5104	
ORF-0971		Replication protein A (fragment)	−1	922408	922743	336	0.5060	Phage-like
ORF-0972		Transposase, IS*Kki1* ORF B	−2	922761	923327	567	0.3351	Transposon
ORF-0973		Transposase, IS*Kki1* ORF A	−2	923355	923591	237	0.4093	Transposon
ORF-0974		Conserved membrane protein of unknown function	−2	923856	924728	873	0.5074	Core genome
ORF-0975		Conserved protein of unknown function	3	924783	925094	312	0.4679	Core genome
ORF-0976	*fixS*	Cytochrome oxidase maturation protein, cbb_3 type	1	925096	925272	177	0.4407	Core genome
ORF-0977		Major facilitator superfamily MFS_1 transporter	2	925229	926548	1,320	0.5136	Core genome

plete and undisrupted (Fig. 1); this confirms that the resistance region of KWG1 has been inserted into this putative exonuclease.

The resistance region appears as a complex structure combining elements of plasmids and transposons. Its 5′ part (ORFs 921 to 924) is nearly (99%) identical to the *repC-sul2-strAB-rcr2*Δ cluster of pSRC15 and other similar plasmids found in Gram-negative bacteria (12) (Fig. 1). The *sul2-strAB-rcr2*Δ cluster is supposed to result from the insertion of transposon Tn*5393c* into a CR2-*sul2* region (12). Interestingly, a fragment of mobile element CR2 (*rcr2*Δ, ORF 2255) is found adjacent to the second copy of *strAB* (ORFs 2253 and 930) (Table 1; Fig. 1). Thus, the cluster of genes described by Yau et al. is present in two pieces separated by the central part carrying the bla_{TEM} gene (Fig. 1) (12). The 3′ part of the resistance region (ORFs 2255 to 942) results from the insertion of transposon Tn*10* (flanked by the 9-bp repeat CCCTGATGA and 23-bp 5′ terminal IRs) (13) into a Tn*5393*-like transposon (Tn*5393c*) (Fig. 1) (14). Finally, the central part carrying the bla_{TEM} gene from ORF 925 to ORF 929 is substantially similar (98% over 82% coverage) to a small bla_{TEM-1}-bearing plasmid in *Haemophilus influenzae* (pA1606; GenBank accession number

JQ611726), as well as similar in organization (*rep*, replication gene; bla_{TEM-1}, TEM-1 β-lactamase gene; *pre*, plasmid recombination enzyme gene; *tnpR*, Tn*3*-like transposon resolvase gene) (Fig. 1) (15). However, unlike pA1606, the *tnpR* resolvase gene of KWG1 (ORF 926) and its *res* sites (I, II, and III) display the closest similarity to those of transposon Tn*2a* (16); moreover, the bla_{TEM-1} gene (ORF 925) is the TEM-1c variant (17), while pA1606 harbors the TEM-1b variant.

Fourteen contigs of the bla_{TEM}-harboring KKC2005004457 plasmid sequencing project (GenBank accession number AMPT00000000), with lengths ranging from 37 to 1,684 bases, are highly (99 to 100%) similar to the whole resistance region (18). However, because of the heavily fragmented nature of these contigs, we cannot tell if the architecture of this *K. kingae* resistance plasmid is similar to that of the KWG1 resistance region.

Two contigs of the β-lactamase-producing *K. kingae* KK247 strain sequencing project (GenBank accession number CCJT00000000) (19) are similar to parts of the ICE: contig 33 with the resistance region (98%) and contig 22 with the MPF_T cluster and the 3′ end of the resistance region (99%) (Fig. 1).

The strong similarity observed between the ICE and contigs of *K. kingae* strains carrying resistance plasmids suggests that transitions between the episomal and plasmidic forms of bla_{TEM}-encoding mobile genetic elements in this species do exist. This observation is in line with recent work that blurs the distinction between ICEs and plasmids (20).

Although no circularized complete sequence of a *K. kingae* resistance plasmid harboring the bla_{TEM} gene is available to date, we may hypothesize the history of the insertion process with a reasonable degree of confidence (Fig. 1).

First, a small bla_{TEM}-harboring plasmid, similar (but not identical) to pA1606, inserted in a larger region associated with resistance to tetracycline, sulfonamides, and streptomycin, near *strAB*, with duplication of these genes. Second, the whole resistance region is later inserted in a larger MPF_T conjugative plasmid, causing disruption of a *rdgC*-like gene. Finally, the conjugative plasmid, or a part of it, inserted itself into the chromosome at Met-tRNA via a phage-like integration process. The precise order of these three events is only postulated and may have differed.

The fact that a similar plasmid is also inserted into the chromosome of *K. denitrificans* suggests that it is an integrative element of the *Kingella* genus. However, this plasmid inserted into another tRNA (Asn-tRNA) with a different integrase lacks several insertion sequences (IS*Nme1* and IS*Kki1*) and does not carry any resistance gene. Recombination and transposition events have thus modified the architecture of this mobile genetic element during its transfer from one species to another.

Interestingly, KK247, described as a strain with plasmid-borne bla_{TEM}, displays significant sequence similarity and a similar architecture for two of its contigs, suggesting that the ICE still exists in its plasmid form in *K. kingae* (19).

As KK247 belongs to clone A, a clone composed mainly of oropharyngeal commensal isolates different from the one to which KWG1 belongs, and as we have previously shown that some *K. kingae* strains belonging to clone A also carry an integrated bla_{TEM} gene on their chromosome, we can conclude that the integration process of the resistance plasmid occurred at least twice in different phylogenetic groups (21).

Episomal integration confers on bacteria the advantage that resistance to antimicrobials is automatically transmitted to daughter cells without the need for plasmid replication. In the era of massive antibiotic use, bacteria harboring stable mechanisms of resistance to various antimicrobials have a selective advantage in colonizing the oropharynxes of children frequently treated with antibiotics. Strains of *K. kingae* clone A have been associated with asymptomatic carriage in Israel and are rarely involved in invasive infections (22). Conversely, KWG1 belongs to a clone involved in osteoarticular infections. The fact that the same plasmid can insert itself into different genetic backgrounds of *Kingella* raises concerns that another, more virulent, clone may also integrate the resistance genes in the future.

Nucleotide sequence accession number. The genome project described here was deposited in the European Nucleotide Archive under accession number LN869922.

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