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Plasmid-Mediated Antibiotic Resistance and Virulence in Gram-negatives: the *Klebsiella pneumoniae* Paradigm

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Summary

Plasmids harbor genes coding for specific functions including virulence factors and antibiotic resistance that permit bacteria to survive the hostile environment found in the host and resist treatment. Together with other genetic elements such as integrons and transposons, and using a variety of mechanisms, plasmids participate in the dissemination of these traits resulting in the virtual elimination of barriers among different kinds of bacteria. In this article we review the current information about physiology and role in virulence and antibiotic resistance of plasmids from the gram-negative opportunistic pathogen *Klebsiella pneumoniae*. This bacterium has acquired multidrug resistance and is the causative agent of serious communityand hospital-acquired infections. It is also included in the recently defined ESKAPE group of bacteria that cause most of US hospital infections.

Keywords

eskape; gram-negative; xer recombination; Klebsiella

The study of plasmids and their biology has had a decisive impact in the advance of molecular genetics contributing numerous fundamental discoveries beyond the field of plasmid biology (1). Interestingly, the study of plasmids was already well under way before the structure of DNA was known with the experiments that led to the discovery of conjugation and recombination in bacteria using as system the plasmid F, known at that time as “F factor” (2, 3). The continuation of these studies showed that bacterial plasmids are responsible for many of the particular properties of bacteria of medical, industrial, or agricultural interest. Their fundamental role in shaping the characteristics of the host bacteria and their ability to propagate led some authors to propose the somewhat controversial idea that they should be considered as independent organisms (4). The role of plasmids in antibiotic resistance was first recognized in Japan when strains that were

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susceptible or multiresistant were isolated from the same patient during a single epidemic of dysentery. This fact suggested that susceptible strains were becoming multiresistant, not through successive mutational steps, but rather by acquisition of the necessary genetic determinants in a single step. Watanabe and Fukasawa reported that this process was due to transfer of a plasmid (at that time called resistance transfer factor, RTF, or R-factor) that harbored the resistance genes (5, 6). Later it became clear that plasmids were carriers of not only antibiotic resistance genes but also genes or groups of genes that specify properties that are essential or contribute to the virulence of the host bacteria (7-20). Studies during the following few decades permitted to learn in some detail numerous biological characteristics of plasmids, as well as the high diversity of existing plasmids and their association with other genetic mobile elements.

There is currently an epidemic of antibiotic resistant bacterial infections, which has been identified as one of the greatest threats to human health by the World Health Organization (http://www.who.int/drugresistance/activities/wha66_side_event/en/index.html) (21-24). Within this epidemic, a group of pathogens has been individualized and collectively named ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, Klebsiella pneumoniae, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*). These pathogens are the causative agents of the majority of hospital infections because they “escape” the antibiotic treatment by becoming resistant or persistent to antibiotic treatment (21, 25-27). Plasmids play a central role in the dissemination and acquisition of the resistant determinants in these bacteria. In this article we describe aspects of plasmids of *K. pneumoniae*, a representative of problematic gram-negative opportunistic pathogens belonging to the ESKAPE group (28-30), with emphasis on their role in virulence and resistance to antibiotics.

K. pneumoniae is the causative agent of serious community- and hospitalacquired infections including but not limited to urinary tract infections, pneumonia, septicemias, meningitis, and soft tissue infections (31-38). *K. pneumoniae* has also been identified as a causative agent of other less common, yet serious, infections such as liver abscess and invasive syndrome (39, 40), septic arthritis (41), or generalized pustulosis (42), and as the triggering factor in the initiation and development of ankylosing spondylitis and Crohn's disease (43-45). *K. pneumoniae* strains have accumulated plasmids that carry virulence and resistance genes that keep increasing its ability to resist the main antibiotics used for treatment such as cephalosporins, carbapenems, penicillins, aminoglycosides or fluoroquinolones (26, 37, 38, 46, 47).

K. pneumoniae strains usually harbor more than one plasmid, including small high copy number and low copy number plasmids that are usually large. Of all completed genomes so far, the multiresistant strain HS11286, isolated from human sputum, harbors the most plasmids with sizes 1.31, 3.35, 3.75, 105.97, 111.19, and 122.80 kbp, respectively (48). More than 70 *K. pneumoniae* plasmids have been completely sequenced and some have been further analyzed with particular attention to the presence of virulence and resistance genes as well as mobile elements.

Small plasmids

A large group of the plasmids found in *K. pneumoniae* isolates are small, with sizes spanning between less than 2-kbp and 25-kbp (Table 1). Most of these plasmids, which share homology at the replication regions (Fig. 1b), replicate through the ColE1-type mechanism (49-53), are non-self transmissible, and not always encode resistance genes as it is the case of plasmids pKPN2 (54) and pKlebBk17/ 80 (55) that encode a restriction-modification system and a bacteriocin, respectively (Table 1). The backbones of the plasmids within this group have a common general organization consisting of the ColE1-type replication region that includes the genes coding for RNA I and RNA II and in some of them the negative regulator *rom* (or *rop*), a transfer region consisting of *oriT* or this locus accompanied by the genes coding for the remaining relaxosome components (56), and a Xer sitespecific recombination site (Fig.1a). Most probably these plasmids are not confined to *K. pneumoniae* but they are able to replicate and be stably maintained in other *Enterobacteriaceae* or gram-negatives. One of the most thoroughly studied ColE1- type plasmids from *K. pneumoniae* is pJHCMW1 (Table 1), isolated from a neonate with meningitis (38, 57). This plasmid is 11,354-bp long, of which 7,992-bp are Tn1331, a multiresistance transposon that includes aac(6')-Ib, ant(3")-*Ia*, bla_{OXA-9}, and bla_{TEM-1} (58-61). The backbone of pJHCMW1 is composed of a ColE1-type replication region lacking *rom*, a functional *oriT* that mediates mobilization when the remaining components of the relaxosome and the transferosome are supplied in trans by a helper plasmid (74), and a Xer site-specific recombination site named mwr, which has been studied in some detail (see below) (75-78). The *aac(6')-Ib*, *ant(3")-Ia*, and $blaO_{XA-9}$ genes are organized in a region resembling the variable region of integrons (59, 60, 62-64). Tn1331, first found in pJHCMW1 (58), or its variations have been found in plasmids hosted by other gram negatives such as *Enterobacter*, *Salmonella*, *Serratia*, and *Pseudomonas* (37, 58, 63-73). pJHCMW1 was recently used as model of ColE1-type plasmids in microscopy studies to determine the mobility of the molecules inside the cells as well as their location and the implications in partition at the moment of cell division (79). Plasmid molecules were highly mobile but were mainly found located at the poles of the cell because they tend to be excluded from the nucleoid occupied space. In fact, in experiments where the nucleoid-free space was increased by using a *dnaN159*(ts) mutant at the non permissive temperature or by treatment with cephalexin the plasmid molecules occupied all the nucleoid-free space (79). These results confirmed that the pJHCMW1 plasmid molecules freely move and are not specifically targeted to the pole but rather they tend to occupy the nucleoid-free space. The molecules were no forming clusters and occasionally were able to move between poles (79). This tendency to preferentially locate at the nucleoid-free poles ensures that at cell division both daughter cells host the plasmid.

A BLAST analysis of the backbone region of pJHCMW1 shows that the Salmonella Typhimurium plasmid pFPTB1, which includes the $bla_{TEM-135}$ and *tetR/tetA* genes in Tn*3*- and Tn*1721*-related transposons, is the most closely related with identity coverage including the replication region (99% identity) and the Xer site-specific recombination site *fpr*, but leaving a gap between these two regions (nucleotides 1770 and 3081) where the *oriT* and a deficient Xer site-specific recombination site are located in pJHCMW1 (Fig. 2) (80, 81).

Fig. 1b shows the alignment of the replication regions of the seven plasmids listed in Table 1. Plasmid p15S completely contains pColEST258 and therefore they share a common block of DNA including the replication region (82). These two plasmids include the region of Tn1331 that encompasses the genes *tnpA*, *tnpR*, and *aac(6')-Ib* (82). All other replication regions are related but not identical. Since small variations in the nucleotide sequence of the replication regions are sufficient to result in compatible plasmids, only experimental assays will determine whether these plasmids are incompatible. All seven plasmids include a Xer site-specific recombination site consisting of the core recombination site and the accessory sequences where the architectural proteins, usually ArgR and PepA, bind to help formation of the synaptic complex (Fig. 1c). The function of this site, at least in some plasmids, is to maximize stabilization by mediating resolution of multimers, a process that leads to a reduction in the effective number of molecules and results in segregational loss of the plasmid (83-87). Other plasmid-related functions of these sites may include exchange of DNA regions among plasmids (54, 88) as well as mediating the insertion of integrative mobile elements into chromosomes and plasmids (83, 89, 90).

The XerD binding sites, usually the most conserved fraction of Xer recombination target sites (91, 92), are identical in all seven plasmids listed in Table 1 (Fig. 1c). XerC binding sites are more divergent and the central regions are considerably different in sequence and size. While six sites include all 8 nucleotides that are highly conserved in all ARG boxes (Fig. 1c), pJHCMW1 and pH205 include a C instead of a T in one of those positions (arrowhead in Fig. 1c). This substitution has been shown to impair the efficiency of a site to act as target for Xer site-specific recombination (76). In particular, the pJHCMW1 Xer site-specific recombination site, called *mwr*, has been studied in more detail and shows some interesting characteristics. Resolution of dimers harboring this site is inefficient when the *E. coli* host cells are cultured in standard L broth (osmolality 209 (mmol/kg) (75,76).). However, the efficiency of resolution is inversely proportional to the osmolarity of the medium and all molecules appear as monomers when cells are cultured in L broth without NaCl added (osmolality 87 mmol/kg) (78). than ideal interaction of ArgR with the mwr ARG-box at higher osmolarity may, at least in part, be responsible for deficient formation of the synaptic complex; a problem that may be compensated when the cells grow in medium with lower osmolarity (Fig. 3). This compensation seems to occur through an increase in negative supercoiling density (Fig. 3) (78). In vitro recombination experiments suggest that the increase in efficiency of resolution occurs at the level of formation of Holliday junction, rather than at the level of Holliday junction resolution (Fig. 3) (78). It is of interest that numerous Xer site-specific recombination sites with identical deficiency in the ARG box have been detected and in some cases experiments demonstrated that they mediate dimer resolution at low efficiency (81). This fact, taken together with other findings like the presence of Xer site-specific recombination sites flanking *blaOXA* genes in *Acinetobacter* plasmids (90, 93-96) or the presence of different DNA fragments flanked by a Xer recombination site and an *oriT* in otherwise identical plasmids (54, 88) leads to the idea that not all Xer recombination sites stabilize plasmids by multimer resolution but may play other, or additional, roles related to plasmid evolution.

Small plasmids may be less than the ideal vehicle for dissemination of resistance genes because a) some of the genes are located outside gene cassettes or mobile elements reducing the versatility shown by these elements to promote dissemination at the molecular level, and b) the plasmids may lack an *oriT* or possess one but lack all other conjugation functions including the proteins that form the specific relaxosome making dissemination at the cellular level by conjugation less efficient (97). Taken together, these factors must reduce the ability of some genes to be mobilized between molecules and cells. A recent report describes a way to reduce this constrain by cointegration of the small plasmid with another plasmid that can provide the machinery for conjugation. The *K. pneumoniae* plasmid pIP843, which harbors the extended spectrum β–lactamase gene $bla_{\text{CTX-M-17}}$ (98), in spite of including an *oriT* locus was not transferred in mating experiments between the original *K. pneumoniae* isolate and a recipient *E. coli* (98). This result suggested that the presence in the same cells of helper plasmids that provide all necessary components, specific and nonspecific, to mobilize plasmids like pIP843 might not be the most usual situation. However, dissemination of $bla_{\text{CTX-M-17}}$ is dramatically enhanced by its presence in a large conjugative plasmid, pE66An, isolated from an *E. coli* clinical strain (99). Interestingly, pE66An has the structure of a cointegrate formed between an original ~73-kbp plasmid and pIP843 (Fig. 4). The point of cointegration is the pIP843 RNA II gene. Therefore, this event resulted in inactivation of the ColE1-type replicon (Fig. 4), which must have been important for generating a stable large plasmid.

Large plasmids

Other plasmids belonging to diverse incompatibility groups, usually larger than those discussed in the previous section, are found in *K. pneumoniae*. While it was well known that antibiotic resistance as well as virulence genes are housed in several of these plasmids, the interest in studying them has recently increased with the realization they host genes responsible for resistance to last resort antibiotics such as bla_{KPC}, $bla_{\text{NDM-1}}$, and bla_{OXA} (34, 46, 100). The virulence phenotype of a *K. pneumoniae* strain was first associated with the presence of a plasmid when it was determined that the 180-kbp plasmid, pKP100, harbors the genes coding for the aerobactin iron uptake system and the mucoid phenotype (101-104). Iron uptake systems are well-known virulence factors of numerous bacterial pathogens (12, 105-108). Loss of pKP100 resulted in concomitant loss of virulence, and the transfer of a mobilizable derivative of pKP100 resulted in reacquisition of the virulent phenotype (103). In another instance, a 185-kbp plasmid was isolated by conjugation using as donor a *K. pneumoniae* isolate that contains three plasmids. This plasmid includes several genes conferring resistance to β-lactams, kanamycin, neomycin, streptomycin, sulfonamides, and tetracyclines, the genes coding for the aerobactin system, and a gene encoding a 29-Kd protein responsible for the ability of this strain to adhere to intestinal cells (109). The association of the mucoid phenotype and production/utilization of aerobactin, and their relation to virulence has been observed in several but not all studies (101, 110, 111). A 219-kbp virulence plasmid, pLVPK, that carries the genes coding for the aerobactin system and is most probably related to those mentioned above was isolated from a highly virulent clinical isolate of K2 serotype and completely sequenced (112). In fact, it has been suggested that numerous *K. pneumoniae* blood isolates harbor a large virulence plasmid of about 200 kbp that includes the aerobactin system, the ability to express the mucoid

phenotype, and resistance to antimicrobials (112). In addition to aerobactin, other iron uptake systems may be included in these plasmids. In the case of pLVPK, the plasmid also includes two more iron-transport systems: the *iroBCDN* cluster that mediates iron uptake through a catecholate siderophore (113), and an homolog of *fecIRA*, which encodes a Fur-dependent regulatory system for iron uptake (114). However, the FecR encoded by this plasmid is truncated at the C-proximal end and the FecA is active in transport but it is induction inactive (115). Interestingly, incomplete FecIRA systems are commonly found in plasmids in *K. pneumoniae* and are chromosomally mediated in *Enterobacter* (115). BLAST analyses against *K. pneumoniae* sequences using the pLVPK regions encompassing the *iutA* and *iucDCBA*, the *iroBCDN*, and the *fecIRA* genes showed 100% homology to the plasmids pK2044 (116) and pKCTC2242 (117) in all three regions and 99% homology to the plasmids pKN-LS6 (accession number JX442974.1), pKPN-IT (82), and pKPN_CZ (118) in the fecIRA region. A comparison of pLVKP, pK2044, and pKCTC2242 using the MAUVE aligner version in which different colors represent local collinear blocks (LCB) with the location of key genes or clusters is shown in Fig. 5. While pVLKP and pK2044 are the most related including all LCBs in the same location, in pKCTC2242 the LCB that in the other two plasmids includes a ter cluster similar to that found in E. coli O157:H7 (119) is truncated, losing the cluster. Furthermore the LCBs in pKCTC2242 are rearranged with respect to pVLKP and pK2044 (Fig. 5). All three plasmids also include an *rmpA2* homolog and *rmpA*, the genes involved in the mucoid phenotype (41, 102, 104, 112). In addition, three physically linked gene clusters coding for resistance to lead, copper, and silver homologs to other known clusters found in *Ralstonia metallidurans* (120), *E. coli* (121), and *Salmonella* Typhimurium (122), respectively, were found in pLVPK as well as pK2044 and pKCTC2242 (Fig. 5). Several other *K. pneumoniae* plasmids, pKPX-1 (123), pUUH239.2 (124), pKN-LS6 (accession number JX442974), pBK32179 (66), pKPN-IT (82), and pKPN-CZ (118), include highly related but not identical fragments.

Replication of pLVPK most probably occurs through the iteron mechanism as a *repA* homolog located between two sets of iterons was found. The region shows higher than 90% homology to the *K. pneumoniae* plasmids, pKCTC2242 (117), pK2044 (116), and pNDM-MAR (72) and the RepA amino acid sequence show similarity to numerous proteins from plasmids isolated from enterobacteria (112). Another putative replication protein was found in pLVPK with homology to several replicator proteins in the database. In addition homologs to *sopA* and *sopB* strongly suggested the presence of an F plasmid-like partitioning system.

An interesting case is that of pClpk, a 150kbp self transferable plasmid isolated from the *K. pneumoniae* C132-98, a strain that caused critical infections over a twoyear period in a hospital and that is characterized for an unusual thermotolerance (125). *K. pneumoniae* C132-98 harbors at least eight plasmids, 4 of which with a size of 100 kbp or larger. The plasmid pClpk encodes ClpK, an ATPase responsible for the increased heat resistance that characterizes *K. pneumoniae* C132-98 (125). The *clpK* gene was then found in other *K. pneumoniae* strains and in at least another plasmid, the 220 kbp IncFIIk pUUH239.2, which also harbors several resistance genes including $bla_{\text{CTX-M-15}}$ and aac(6')-Ib-cr (124, 126).

Although cases such as that of the small plasmid pJHCMW1 (38, 57, 58, 61) are well known, numerous plasmids including multiple resistance genes are large and self transmissible. This conjugation machinery requires genes coding for the relaxosome, the type IV coupling protein, and the type IV secretion system (127). As illustrated in previous paragraphs the resistance genes may be included in various genetic elements and coexist with other genetic elements and genes coding for resistance to metals or virulence factors.

A well studied multiresistance *K. pneumoniae* plasmid is pMET1, a plasmid isolated form a clinical strain responsible for a high mortality hospital outbreak (37, 128). Its replication was extensively studied by cloning and deletion assays that permitted to identify a 1655-bp replication region that includes *repA*, which codes for a protein belonging to the RepA IncFII superfamily, and an AT-rich sequence likely to include *ori* (128). BLASTn analysis of this region showed extensive homology only with the *Yersinia pestis* plasmid pCRY (129), the *Cronobacter turicensis* plasmid pCTU2 (130) and the C. sakazakii plasmid pESA2 (accession number CP000784). In addition, a pMET1 segment including the type IV secretion system, which is related to those found in the ICE_{Kp1} and the HPI_{ECOR31} (131), and the mobB and mobC genes shared high similarity with the cryptic $_{Yersinia\ pestis}$ plasmid pCRY. The homology shared between the cryptic pCRY and the multidrug resistance pMET1 suggests that the latter plasmid can be replicated and stably maintained in *Yersinia* species, representing a high public health and biodefense threat due to transfer of multiple resistance genes to pathogenic *Yersinia* strains. Absent in pCRY are the pMET1 partition genes *parF* and *parG*. The resistance genes in pMET1 reside in Tn*1331*.2, an 11,042-bp transposon highly related to Tn*1331*, the transposon found in pJHCMW1 (see above). Tn*1331*.2 has a perfect duplication of the 3,047-bp DNA region that includes aac(6')-Ib, *aadA1*, and bla_{OXA-9} (37, 128).

The resistance to antibiotics encoded by some of the large *K. pneumoniae* plasmids plays a central role in enhancing the morbidity and mortality of *K. pneumoniae* due to the advent of the carbapenemases, in particular KPC (*K. pneumoniae* carbapenemase) and NDM-1 (New Delhi metallo-β-lactamase-1), creating a significant public health threat (66, 132, 133). Both bla_{KPC} and bla_{NDM-1} were first detected in *K. pneumoniae* but they are present in other gram-negatives. The emergence of carbapenemases has virtually eliminated the possibility of using β-lactams to treat multidrug resistance gram-negative infections (134). Furthermore, the presence of genes coding for carbapenemases is usually accompanied by others that code for resistance to other β-lactams, aminoglycosides, and fluoroquinolones, which leaves few treatment options (34, 100, 135). In particular the common localization of carbapenemase-coding genes in plasmids has renewed the interest in their study and numerous large plasmids coding for carbapenemases (KPC, VIM, NDM, IMP, and OXA types) and CTX-M type extended spectrum β-lactamases have been fully sequenced (132). Since there is consensus that currently we are witnessing a convergence of two plasmid-driven epidemics, one of bla_{KPC} and the other of bla_{NDM} carrying *K. pneumoniae* (100, 134, 136-138), in the following paragraphs we describe some representative characteristics about plasmids including bla_{NDM} and bla_{KPC} genes.

The carbapenemase NDM-1 is a broad-spectrum metallo-β-lactamase that mediates inactivation of nearly all β-lactams with the exception of aztreonam. It was first identified in

a *K. pneumoniae* isolate from a urinary tract infection (139) and has rapidly disseminated among *Enterobacteriaceae* and *Acinetobacter* spp (140). Plasmids carrying $bla_{\text{NDM-1}}$ belonging to different incompatibility groups have already been isolated. Fig. 6 shows a comparative diagram of sequenced *K. pneumoniae* plasmids that harbor this gene. Although plasmids carrying the gene can be largely unrelated belonging to different incompatibility groups (Fig. 6a), the immediate environment of $bla_{\text{NDM-1}}$ is highly related in all plasmids (Fig. 6b). The blaKPC gene is found as part of the 10-kbp transposon Tn*4401*, which is present in numerous multiresistance plasmids belonging to different incompatibility groups. It may be worth mentioning that Tn*4401* has not yet been found in any chromosome. The large number of different conjugative plasmids allowed the rapid and successful spreading of bla_{KPC} to other gram-negatives. Of special interest is the recent report of the IncI plasmid pBK15692 in which Tn*4401* has inserted within the *tnpA* gene of Tn1331 (Fig. 7) creating a transposon with the capability to confer resistance to virtually all β-lactams and aminoglycosides. The dissemination of pBK15692 will increase the already complicated landscape of gram-negative infections.

Concluding remarks

Most interactions between humans and bacteria do not result in disease and many of them are beneficial for one or both interacting partners. When the interaction results in disease, the relation between humans and pathogenic bacteria has been one resembling an arms race through evolution. As the human body was developing and evolving numerous strategies to defend against bacterial infections, bacteria were doing the same to counter those defenses that limit or prevent the establishment of invading bacteria. In addition an artificial defense that humans have counted for a few decades is the utilization of antibiotics. The evading strategies that permit bacteria to colonize or cause damage to the host are known as virulence factors. The presence or absence of a virulence factor can determine whether a bacterium behaves as a pathogen or not in normal conditions. Numerous virulence factors as well as antibiotic resistance genes are usually part of plasmids or genetic elements located in plasmids that have the capability to disseminate at the molecular level such as integrons or transposons. This intense activity permits the generation of new variations of plasmid molecules that can accumulate genetic determinants for several virulence factors and resistance. This dissemination in combination with the tremendous tendency that plasmids have to disseminate at the cellular level results in the virtual elimination of barriers among different kinds of bacteria permitting these genes to reach nearly all bacteria (141, 142). Plasmids play an essential role in this fluid situation in which genes behave as a pool being able to reach any bacteria transforming them from friend into foe or from susceptible into resistant. This chapter illustrates the rich variety of plasmids that can harbor numerous virulence factors and resistance genes in *K. pneumoniae*. However, careful examination of the existing knowledge shows that we are still barely scratching the surface. There is a wealth of information that still needs to be acquired about these plasmids. Their study using the most modern technologies will enhance the possibilities to design new strategies to deal with emerging infectious diseases that represent a serious threat to human health.

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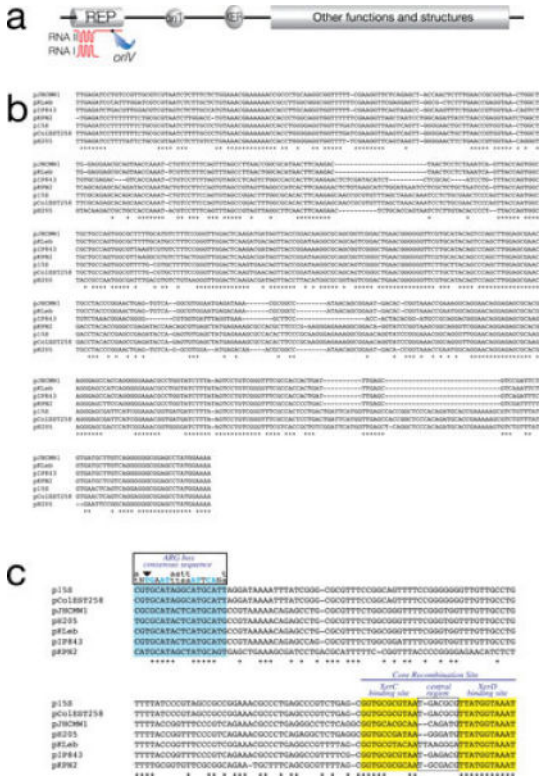

Fig. 1.

Small plasmids. a, General genetic organization of small ColE1-type plasmids from *K. pneumoniae* and other *Enterobacteriaceae*. b, Alignment of the nucleotide sequences of the replication regions of *K. pneumoniae* ColE1-type plasmids using CLUSTAL W (143). c, Alignment of the nucleotide sequences of Xer site-specific recombination sites of K. pneumoniae ColE1-type plasmids using CLUSTAL W. The ARG box, XerC and XerD binding sites are shown in color and the central regions are boxed. Blue capital letters indicate the most important conserved nucleotides in the ARG box. Downward pointing arrowhead shows the conserved T nucleotide that its found substituted by a C in several Xer site-specific recombination sites (76, 81, 88).

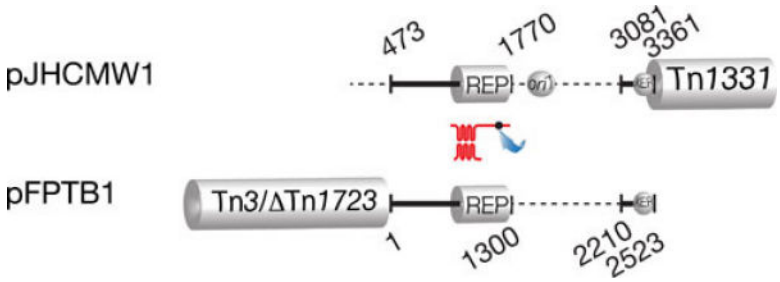

Fig. 2.

Comparison of pFPBT1 and pJHCMW1. The black lines, which represent regions of homology (coordinates 463-3361 in pJHCMW1), are drawn at scale. The Tn*3*-like transposons, Tn*1331* and Tn*3*/DeltaTn*1723*, as well as the dots indicating *oriT* and the Xer target sites are shown at the correct locations but are not drawn to scale. The replication regions (REP) share 97% homology. The numbers indicate the coordinates in the GenBank database (pJHCMW1, accession number AF479774; pFPBT1, accession number AJ634602). The location of the similar but not identical Xer site-specific recombination sites (81) is indicated.

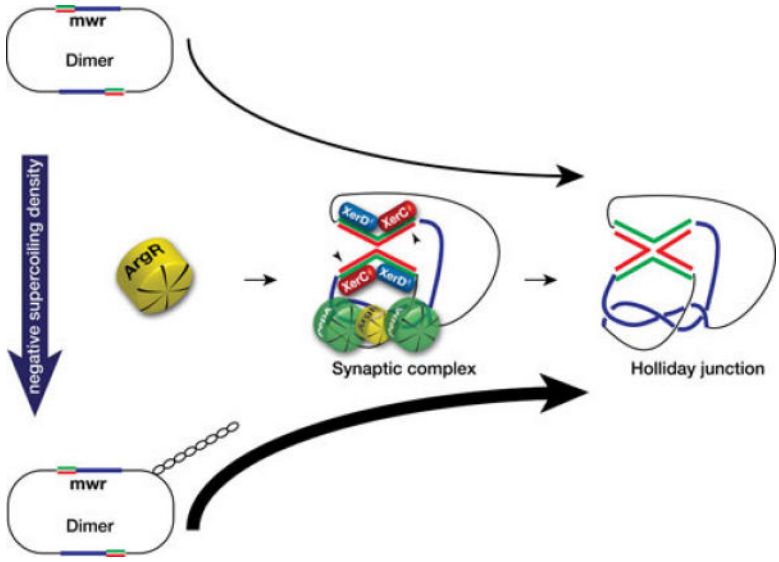

Fig. 3.

Effect of changes in osmolarity of the culture medium on Xer site-specific recombination at *mwr*. Schematic representation of the possible chain of events that lead to a higher efficiency of Xer site-specific recombination at the *K. pneumoniae* plasmid pJHCMW1 site mwr. A decrease in the NaCl concentration in the growth medium (L broth containing 0.5% NaCl added to no NaCl added) is correlated with an increase in supercoiling density, which facilitates interaction of ArgR with the substandard *mwr* ARG box leading to a more efficient formation of a productive synaptic complex and Holliday junction (78). Molecular models of the interwrapped synaptic complex are available at the following references (146-148). The two strands are shown only in the core recombination site (red and green lines), blue lines represent the accessory sequences.

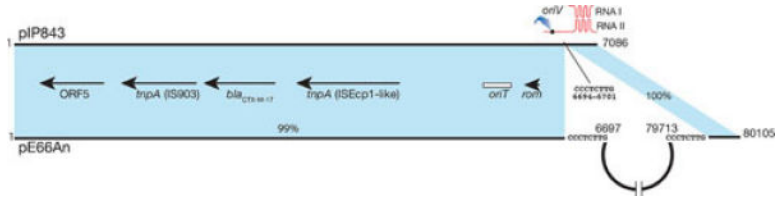

Fig. 4.

Genetic maps comparison of the *K. pneumoniae* pIP843 and the E. coli pE66An. The shadowed areas show regions of homology (6681/6701 identities and 6 gaps in the region with 99% homology). The ColE1-type replication region is schematically shown on top of the pIP843 map. The semicircle in pE66An represents the region encompassing nucleotides 6697 – 79713.

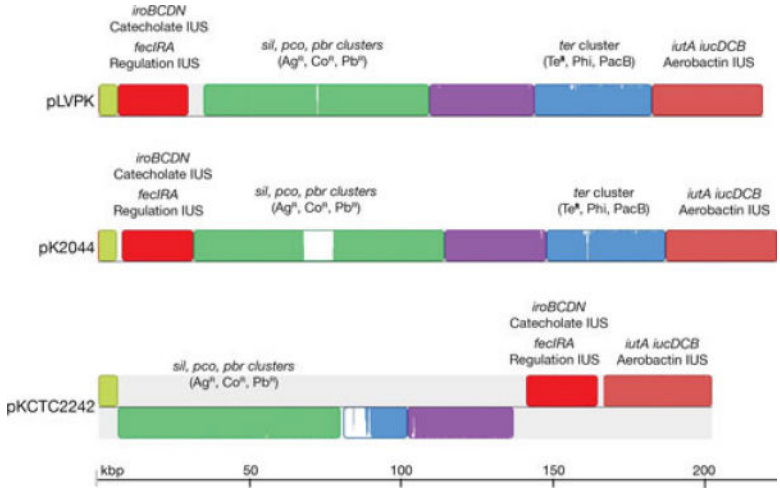

Fig. 5.

Multiple alignment of pLVPK, pK2044 and pKCTC2242. The nucleotide sequences of pLVPK (accession number AY378100.1)(112), pK2044 (accession number AP006726.1) (116), and pKCTC2242 (accession number CP002911.1)(117) were compared using the MAUVE aligner version 2.3.1 (149). Different colors represent local LCBs. Inside each block there is a similarity profile of the sequence, the height corresponds to the average level of conservation. Completely white areas are not aligned and most probably contain sequences specific to the particular molecule. In pKCTC2242 the LCBs drawn below the black line are inverted with respect to their homologs in pLVPK and pK2044. Some genes or clusters present in these blocks are identified by name. The *terZ* gene has been reported as "truncated" (112). The truncation is a consequence of an extra T in the sequence that could also be a sequencing error. The *terBCDE* genes are sufficient for the tellurite resistance phenotype (Te^R). The *ter* cluster is also responsible for the phage inhibition (Phi) and colicin resistance (PacB) phenotypes (150). Copper (*pco*), silver (*sil*), lead (*pbr*), and tellurite (*ter*) resistance related genes; IUS, iron uptake system.

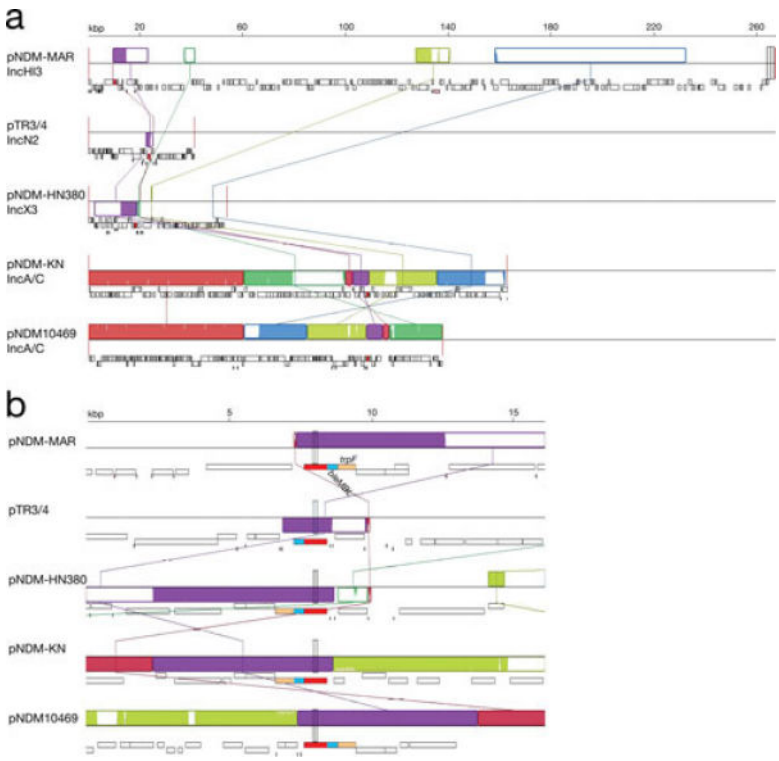

Fig. 6.

Multiple alignment of pNDM-MAR, pTR3/4, pNDM-HN380, pNDM-KN, and pNDM10469. The nucleotide sequences of pNDM-MAR (accession number JN420336) (72), pTR3/4 (accession number JQ349086) (151), pNDM-HN380 (accession number JX104760) (152), pNDM-KN (accession number JN157804) (153), and pNDM10469 (accession number JN861072) were compared using the MAUVE aligner version 2.3.1 (149). The $bla_{\text{NDM-1}}$ gene is represented in red, genes ble_{MBL} and *trpF* are represented in light blue and light brown, respectively. Plasmids pTR3 and pTR4, originally thought to be similar but not identical were later proved to be identical and renamed pTR3/4 (151). a. The comparison of the complete nucleotide sequence is shown with LCBs represented in blocks of different colors. b. Zoom in the region including the $bla_{\text{NDM-1}}$ gene.

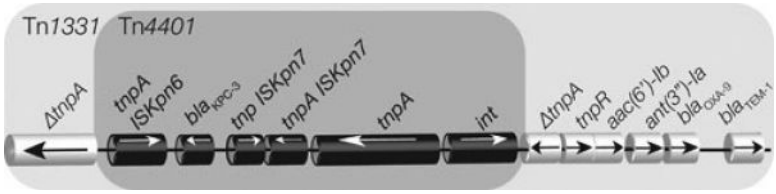

Fig. 7.

Genetic map of the Tn*1331*: :Tn*4401* region in the $_{\text{K. pneumoniae}}$ plasmid pBK692.

Table 1

Completely sequenced ColE1-type plasmids of *K. pneumoniae*

Plasmid[1]	Size (bp)	Relevant genotype	Accession number	Reference
pKPN2	4,196	*kpn2kIR, kpn2kIM*	AF300473	(54)
pIP843	7,086	*bla* $_{\text{CTX-M-17}}$	AY033516	(98)
pH205	8,197	*bla* $_{\text{CMY-36}}$	EU331426	(154)
pKlebB-k17/80	5,258	*kba, kbi*	AF156893	(55)
pJHCMWl	11,354	*aac(6′)-Ib, ant(3″)-Ia, bla* $_{\text{OXA-9}}$ *, bla* $_{\text{TEM}}$	AF479774	(61)
pS15	24,296	*aac(6')-Ib, bla*$_{\text{KPC-2}}$, cloacin, immunity protein	FJ223606	(69)
pColEST258	13,636	*aac(6')-Ib*, cloacin, immunity protein	JN247853	(82)

kpn2kIR, kpn2kIM, Type II restriction-modification system; *kba, kbi*, Klebicin B (a bacteriocin) and immunity.

[1] Plasmids that have been subjected to characterization studies beyond just sequencing