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The intrinsic resistome of *Klebsiella pneumoniae*

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ABSTRACT

Molecular epidemiology studies aiming at understanding the acquisition of antimicrobial resistance by clinical isolates of *Klebsiella pneumoniae* are regularly published; however, information on the genes that contribute to its characteristic phenotype of resistance to antibiotics (intrinsic resistome) is scarce. To fill this gap, a *K. pneumoniae* transposon mutant library was screened and 171 mutants presenting changes in their susceptibility to antibiotics were selected, in which the transposon insertion site was determined in 75. Twenty-seven mutants for which insertion points had been previously identified were included in the analysis. A total of 102 mutants were selected for further studies. In 70 mutants the transposon was inserted in a gene with a known function, whilst in 19 the insertion occurred in genes encoding proteins with unknown functions and 13 insertions occurred in intergenic regions. Moreover, 87 of the insertions were localised in the chromosome, with 15 insertions located in the two plasmids carried by this strain. Whereas some of the mutated genes are already known to be involved in antimicrobial resistance (*ampG, acrB, tolC*), several of them are involved in regular processes of bacterial physiology, including *K. pneumoniae* virulence. Together with results published for other organisms, these results support that determinants involved in basic processes of bacterial physiology may contribute to antimicrobial resistance. These findings also indicate that, besides acquired resistance genes, plasmids may harbour other genes belonging to their backbone that can also be involved in resistance.

1. Introduction

Klebsiella pneumoniae is a member of the so-called 'ESKAPE' group that includes six of the most significant antimicrobial-resistant microorganisms (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) responsible of nosocomial infections all around the world [1].

Klebsiella pneumoniae presents different chromosomally-encoded antimicrobial resistance determinants capable of reducing the susceptibility of this micro-organism to different antibiotics, some of which have been transferred to other bacteria. Among them, we can highlight the SHV β-lactamase [2], the fosfomycin resistance gene *fosA* [3] and the nalidixic acid efflux pump OqxAB [4]. In addition, *K. pneumoniae* can also acquire resistance by mutation or through the acquisition of antimicrobial resistance genes by means of horizontal gene transfer. Indeed, it has been suggested that *K. pneumoniae* can play an important role in antimicrobial resistance transfer to and from other micro-organisms, including β-lactamases such as KPC, OXA or NDM [5], being thus a hub for such transmission. Consequently, tracking the genes that contribute to the characteristic phenotype of resistance is an interesting topic in order to predict the future evolution of the pathogen itself and for identifying putative resistance genes whose transfer might confer resistance to a heterologous host.

Indeed, in the last years a number of studies aimed at identifying genes involved in the characteristic antimicrobial resistance of different pathogens, which has been dubbed as the intrinsic resistome [6,7], have been published [7–11]. The intrinsic resistome has been defined as 'the ensemble of chromosomal genes that are involved in intrinsic resistance and whose presence in strains of a bacterial species is independent of previous antibiotic exposure and is not due to horizontal gene transfer' [7]. The study of transposon-tagged libraries can provide information on two aspects of antimicrobial resistance. Inactivation of some genes makes bacteria more susceptible to antibiotics. These genes form the bona fide intrinsic resistome because their presence make bacteria more

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resistant to antimicrobials and they could be good candidates as targets for inhibitors of resistance to be used in combination with antibiotics [12]. In addition, their transfer might confer resistance to a heterologous host, and surveillance of this possibility should be taken into consideration. The other category is formed by those genes whose inactivation reduces antimicrobial susceptibility. This category is important for predicting mutation-driven resistance. In the present work, we have explored these phenotypes in a transposon-tagged library from *K. pneumoniae* strain 52.145. The results provide novel information on the intrinsic resistome and the potential routes of evolution towards resistance of this bacterial pathogen, which can be used both for predicting novel mechanisms of resistance and for identifying targets for the development of antibiotic potentiators able to increase the therapeutic window of the potentiated antibiotics.

2. Materials and methods

2.1. Strains, culture media and antibiotics

Clinical strain *K. pneumoniae* 52.145 (serotype O1:K2), whose sequence is available at https://www.ncbi.nlm.nih.gov/nuccore/ (reference sequence: chromosome, **NZ_FO834906.1**; plasmid II, **NZ_FO834905.1**; and plasmid I, **NZ_FO834904.1**), and the transposon mutant library derived from this strain (56 96-well plates with 5320 independent mutants) have been described previosuly [13].

The different strains were grown regularly at 37°C in Luria–Bertani (LB) medium. For antimicrobial susceptibility assays, strains were grown on Mueller–Hinton (MH) agar [14].

2.2. Antimicrobial susceptibility testing

A first determination of the minimum inhibitory concentrations (MICs) of *K. pneumoniae* 52.145 and screening of the transposon mutant library was performed on MH agar plates by two-fold dilution for the following antibiotics: gentamicin; erythromycin; tetracycline; chloramphenicol; ciprofloxacin; imipenem; cefoxitin; cefazolin; and ceftazidime.

A more precise determination of the susceptibility to antibiotics of the mutants selected in the screening was determined on MH agar plates using MIC Test Strips (Liofilchem®, Roseto degli Abruzzi, Italy) of the same antibiotics, with the exception of cefazolin for which MIC Test Strips were not available.

2.3. Screening of mutants presenting different levels of antimicrobial susceptibility

For the screening, each independent clone from the library was inoculated into 50 µL of LB medium in 384-well plates and was grown overnight at 37°C. Then, a 384-pin replicator was used to inoculate MH agar plates. The replicator inoculates ca. 0.2 µL on each plate and consequently the chances that the inoculated cultures contain spontaneous mutants are low. The plates contained different concentrations of antibiotics higher and lower than the MICs corresponding to the wild-type strain as determined by doubling dilution. The MICs obtained by doubling dilution were as follows: gentamicin, 0.25 µg/mL; erythromycin, 32 µg/mL; tetracycline, 2 µg/mL; chloramphenicol, 2 µg/mL; ciprofloxacin, 0.06 µg/mL; imipenem, 0.125 µg/mL; cefoxitin, 8 µg/mL; cefazolin, 2 µg/mL; and ceftazidime, 8 µg/mL. In all cases, each of the plates contained a spot of the wild-type strain as a control of susceptibility to antibiotics. Plates were incubated overnight at 37°C. Each spot (mutant) was annotated as more or less resistant (growth or not growth) in comparison with the wild-type strain present on the same plate. To avoid false positives, the mutants selected from the first screening were submitted to a second screening under the same conditions, and 436 mutants presenting different susceptibility to antibiotics were chosen for future work. These mutants were used to generate five 96-wells plates, and a third screening was performed with these plates, reducing the number of mutants presenting consistent changes in their susceptibility phenotype to 220. Standard double-dilution tests do not allow an accurate discrimination of small MIC differences. However, these differences can be easily detected by Etest since the strips used for MIC determination contain a linear gradient of antibiotics concentrations. Consequently, the MICs of the different antibiotics for the selected mutants were determined using MIC Test Strips (Liofilchem®). Hence, note that the MICs obtained by doubling dilution and using MIC Test Strips are not exactly the same.

2.4. Identification of the point of insertion of the transposon

Genes interrupted by the transposon (Tn*5RL27*) were identified by inverse PCR as described previously [7]. Briefly, each selected mutant was grown in LB medium at 37°C overnight. Genomic DNA of each mutant was obtained using a GNOME® DNA Isolation Kit (MP Biomedicals, Santa Ana, CA). Genomic DNA was digested with *Eco*RV or *Pst*I (New England Biolabs Inc., Ipswich, MA) during 7 h at 37°C. Digested DNA was precipitated (1/10 of sodium acetate 3 M and 1 volume of isopropanol, then washed with ethanol 75%) and 500 ng were circulated with 400 U of T4 ligase (New England Biolabs Inc.) in a volume of 200 µL at 16°C overnight. All ligated DNA was precipitated (as described above) and was used as template for inverse PCR using an Expand™ Long Template PCR System (Roche, Basel, Switzerland), buffer no. 3 and the primers tpnRL17-2 (5′-TGTAACGCACTGAGAAGCCCTTAGAGC-3′) and tpnRL13-3 (5′-CGATTCAGGCCTGGTATGAGTCAGC-3′). The PCR program consisted of 1 denaturation step at 95°C for 2 min, 30 amplification cycles of 95°C for 10 s, 55°C for 30 s and 68°C for 6 min, and a final extension step at 68°C for 7 min.

PCR products were purified using a QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany) or from gel (when more than one PCR product was obtained) with an Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Addison, IL) and were sequenced at Macrogen (Seoul, South Korea), STAB VIDA (Caparica, Portugal) or Parque Científico de Madrid (Madrid, Spain). To identify the gene interrupted by the transposon in each of the mutants, a BLAST search of the NCBI's Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the sequence obtained was performed. The insertion point of several mutants was confirmed by amplifying the region of interest using the primers described in Table 1 and further sequencing of the amplicons.

3. Results and discussion

Screening of transposon libraries has been used in *K. pneumoniae* to identify genes involved in virulence [13], biofilm formation [15] and intestinal colonisation [16] of this bacterial pathogen. One study based on the estimation of mutant depletion upon exposure to three antimicrobials by transposon-directed insertion-site sequencing has also been published [17]. However, the use of insertion libraries to gain information on genes involved in intrinsic resistance, as well as genes whose mutation may lead to antimicrobial resistance in this pathogen, has not been particularly explored.

Consequently, in this study a *K. pneumoniae* transposon mutant library containing 5320 independent mutants was screened by comparing the antimicrobial susceptibility of each of the mutants with that of the wild-type strain. To that goal, *K. pneumoniae* 52.145, a reference strain with serotype O1:K2 and sequence type 66 (ST66), was used. Hypervirulent *K. pneumoniae* isolates may

Table 1
Oligonucleotide primers used in this work

Gene	Primer	Sequence 5′→3′	Description/use
cadC	cadC2	ATGGAACTACTCGGTTAACC	Amplification of a fragment of 1540 bp (wt) or 3357 bp (with transposon)
	cadC3	ATGCTACAACCTGTTGTTCG	
mgl	mgl1	ATGAACCCACGCAAACCC	Amplification of a fragment of 1014 bp (wt) or 2831 bp (with transposon)
	mgl3	TCATATAACAAAGGGCGACG	
yfgA	yfgA3	ATGAATACTGAAGCCACTC	Amplification of a fragment of 996 bp (wt) or 2813 bp (with transposon)
	yfgA4	TTACTGCGCCGGTGATGATTC	
waaL	waaL1	AAGATCTACTATTTAGCAGG	Amplification of a fragment of 1094 bp (wt) or 2911 bp (with transposon)
	waaL2	AAGATGCTAAGGATTAATGC	

wt, wild-type.

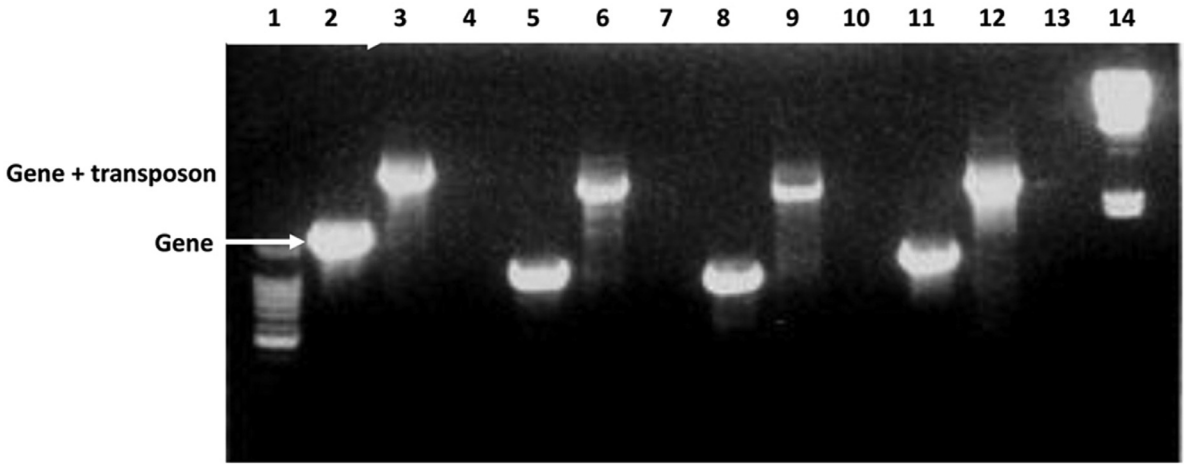

Fig. 1. Confirmation of the location of the transposon in a set of mutants. Presence of the transposon was confirmed by PCR in four mutants. Lanes 2, 5, 8 and 11, *Klebsiella pneumoniae* 52.145 (wild-type); lanes 3, 6, 9 and 12, mutants P006G10, P012G4, P012H7 and P025H3, respectively; lanes 4, 7, 10 and 13, control without DNA. Lanes 1 and 14 correspond to DNA markers (100 bp and λ DNA-*Hin*dIII) (New England Biolabs Inc., Ipswich, MA), respectively.

belong to serotypes K1 and K2. Whilst most serotype K1 isolates belong to the multilocus sequence typing (MLST) ST23 category, *K. pneumoniae* K2 isolates present diverse MLST types including ST65, ST66, ST86, ST374, ST375 and ST380 among others [18]. The chromosome and the two plasmids of *K. pneumoniae* strain 52.145 have been sequenced (see above) and it has been shown that this isolate contains the virulence factors of hypervirulent *K. pneumoniae* isolates [19], including the large virulence plasmid harbouring the aerobactin cluster and the regulator of the mucoid phenotype [20,21]. The MICs of different antibiotics belonging to different families, with different resistance mechanisms and affecting different bacterial processes (e.g. DNA synthesis, ciprofloxacin; protein synthesis, gentamicin, erythromycin, tetracycline and chloramphenicol; and cell wall synthesis, imipenem, cefoxitin, cefazolin and ceftazidime), against the wild-type strain *K. pneumoniae* 52.145 was determined by doubling dilution on MH agar plates. Taking into consideration the MIC of the wild-type parental strain as determined by doubling dilution, the antibiotic selective concentrations (see Section 2.3) were 2 × MIC for detecting mutations that increase resistance and 0.5 × MIC for detecting hypersusceptible mutants. In some cases, when these antibiotic concentrations did not allow an accurate discrimination of the mutants, other concentrations were also tested. Among the 220 mutants selected in the screening, 171 presented a consistent phenotype following MIC determination by Etest. Although changes in susceptibility to antibiotics were low on different occasions, these changes have been confirmed after three sequential screenings (see Section 2.3) and MIC determination by Etest, which allow a precise discrimination of small MIC changes. Various works have discussed the relevance of small changes in susceptibility to antibiotics in the development of clinically relevant resistance [22], including the MIC creep observed in bacterial pathogens [23]. Consequently, instead of clinical breakpoints, we used the operational definition of antimicrobial resistance as discussed previously [24]. The current results provide information on mutations producing such small changes that may remain obscure in classical molecular epidemiology studies on antimicrobial resistance but that can be the prelude for the acquisition of resistance [24]. Among the studied mutants, the position of the transposon has been previously determined in 27 of them [13]. For the other 144 mutants, the position of the transposon was analysed by inverse PCR. Among them, the gene where the transposon was located was identified in 75 of the mutants. Previous whole-genome sequencing of ca. 200 mutants from this library has shown that each of the mutants contains just one copy of the transposon, without any further mutation [13]. Consequently, only one gene should be inactivated in each of the studied mutants, and the inactivated gene should solely be responsible for the observed phenotype. Together with the 27 previously mapped mutants [13] that were included in this study, 102 mutants were selected for further analysis (Supplementary Table S1). For some mutants, the site of insertion of the transposon was confirmed by PCR using the primers shown in Table 1, which flank the positions where the transposon has been inserted. In all cases, this analysis confirmed the results of the inverse PCR analysis (Fig. 1), supporting the reliability of the results. In two cases in which the phenotype was strong and we did not get a positive inverse PCR amplification, the genes interrupted by the transposon were determined upon sequencing the complete genomes of the mutants at Parque Cientifico de Madrid as described previosuly [25]. In agreement with previous results, no further mutation besides the transposon insertion was observed in either of these two mutants.

In 87 of the selected mutants the transposon was localised on the chromosome, whilst 15 insertions were in genes present on either of the two plasmids present in *K. pneumoniae* 52.145 (11

mutants in plasmid I and 4 mutants in plasmid II). As shown in Supplementary Table S1, most mutants presented changes in susceptibility to antibiotics belonging to different structural families. These results are in line with those published for other organisms [6–9,26–28] and indicate that the intrinsic resistance of bacterial pathogens is the result of the action of several elements encoded in the microbial genome. For some of the mutants, the reason for the observed phenotype was difficult to infer, particularly in cases in which the interrupted gene codes for unknown proteins ($n = 19$) or when the transposon was integrated between two genes ($n = 13$). For the latter, a polar effect on transcription of the genes localised downstream might be suggested. This is the case of the mutants in which the transposon is located upstream *phoE* (P002C3) or *psiE* (P011C10), genes for which a role in resistance to toxic compounds has been suggested, as well as the mutant P047H5 in which the transposon is located upstream of the gene encoding an O-antigen transporter (Supplementary Table S1).

Other cases in which the effect is likely indirect are the insertions in *mutS* and *recA*. It is known that the mutation of *mutS* (P049B3), a key component of the DNA mismatch repair system, increases the mutation frequency and is critical for the acquisition of antimicrobial resistance [29]. Hence, it is quite likely that other mutations besides *mutS* are involved in the observed phenotype. Something similar might happen with *recA* (P030E7). Besides being involved in susceptibility to quinolones, RecA has a key role in homologous recombination and in SOS-driven mutagenesis [30]. Similarly, mutations as those of the P009C4, P019F8, P012G4 and P041F4 mutants, which are located in genes encoding transcriptional regulators may affect antimicrobial susceptibility and the inflammatory response [13] because of changes in the level of expression of the genes they regulate.

The first step required for the activity of an antibiotic is its interaction with the cell envelope and/or its entrance into the bacterial cell. Consistent with this situation, several mutations occurred in genes involved in different aspects of the physiology of bacterial cell envelopes, such as its synthesis, maintenance of the cell wall, and processes in which the envelope is of relevance such as adhesion and biofilm formation (P005E3, P012A10, P018G12, P021E9, P025F5, P043A12 and P049G10). In addition, a high percentage of the interrupted genes code for elements involved in the transport or extrusion of metabolites. It is quite likely that these elements may be involved in the entrance or extrusion of the antibiotics analysed in this article.

Although the mechanisms are not known, several of the genes whose inactivation alters the susceptibility to antibiotics of *K. pneumoniae* have been reported to modify the susceptibility to toxic compounds in different bacteria (Supplementary Table S1). This is particularly relevant for insertions in genes involved in biosynthesis of the cell envelope: among the 14 mutants, 8 of them have been described to be involved in the susceptibility to different antibiotics and 4 more (P005E3, P015F8, P038C6 or P028G2) are involved in the synthesis of lipopolysaccharide, a critical element for the entrance of antibiotics. Altogether, these previously published findings validate the reliability of the current analysis, even when the mutations occur in genes not previously classified as antimicrobial resistance determinants.

Particularly intriguing is the potential role that proteins encoded by genes belonging to the plasmid backbone and not acquired as the consequence of antibiotic selective pressure may have in antimicrobial resistance. This is the case for genes located in plasmid I encoding different proteins (TraC, TraE, TraH, TraM and TraN) of the transfer pili, which is needed for translocation of DNA [31]. The study of mutations in plasmid genes that can be involved in antimicrobial resistance has almost exclusively focused on the evolution of plasmid-encoded antimicrobial resistance determinants [32,33]. However, the results of the current study, and those from other authors [34], indicate that, as happens in the case of chromosomally-encoded housekeeping proteins [7], different elements belonging to the plasmid backbone can contribute to antimicrobial resistance besides acquired antimicrobial resistance determinants.

Noteworthy, some of the genes identified in this work (Supplementary Table S1) have been also identified to be involved in bacterial virulence. Among them, some genes were identified in a previous screening whose purpose was to find factors implicated in the suppression of nuclear factor κB (NF-κB) activation [13]. Other genes, such as *waaL*, *rfbB*, undecaprenyl-phosphate glucose-1-phosphotransferase (*wcaJ*), the multidrug efflux RND transporter permease subunit (*acrB*) and *surA*, were found looking for genes involved in virulence [13,35]. It is worth noting here that AcrAB is a major contributor to intrinsic antimicrobial resistance in Enterobacteriaceae [36,37], which also plays a role in the virulence of these micro-organisms, including *K. pneumoniae* [38]. Altogether these results indicate that antimicrobial resistance and virulence can be tightly interlinked in bacterial pathogens [39].

4. Conclusions

These results indicate that, similar to the situation with other pathogens, the resistome of *K. pneumoniae* comprises a variety of genes belonging to different functional categories, most of them not previously considered to encode antimicrobial resistance determinants. This result reinforces the idea that the characteristic phenotype of antimicrobial resistance of bacterial pathogens is the result of the concerted action of several different elements, which altogether contribute to bacterial robustness and its response to injuries. In addition, finding genes whose inactivation increases the susceptibility to antibiotics of *K. pneumoniae* provides new targets for developing compounds able to increase the efficiency of known antibiotics against this ESKAPE pathogen, whereas the genes whose inactivation decreases antimicrobial susceptibility provide information on novel potential antimicrobial resistance mechanisms.

Finally, we would like to highlight that, similar to the situation regarding chromosomal genes, plasmids may harbour genes as part of their backbone and not acquired as the consequence of antibiotic selective pressure that may contribute to antimicrobial resistance.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.09.012.

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