Duplication of the chromosomal *bla*_{SHV-11} gene in a clinical hypermutable strain of *Klebsiella pneumoniae*

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In a collection of 110 clinical isolates of *Klebsiella pneumoniae*, a single strain, Kp593, was found to exhibit a mutator phenotype with a rifampicin mutation frequency 100-fold higher than the modal value for this species. Complementation experiments with the wild-type MutL, one of the main components of the methyl-directed mismatch repair system, allowed the mutator phenotype to be reversed. Sequencing revealed substitution of the conserved residue Lys307 to Arg and site-directed mutagenesis followed by complementation experiments confirmed the critical role of this mutation. The patient infected with Kp593 relapsed a month later and the strain isolated then, Kp869, was identical to Kp593, as verified by PFGE analysis. Phenotypically, Kp869 colonies were more mucoid than those of Kp593, probably due to increased capsule synthesis as shown by electron microscopy. In addition, Kp869 exhibited a 16-fold higher amoxicillin resistance level related to a 36.4 kb tandem duplication encompassing the chromosomal *bla*_{SHV-11} gene, which was unstable *in vitro*. These data suggest that the mutator phenotype found in Kp593/Kp869 is associated with beneficial mutations conferring a selective advantage, such as increased virulence factor production and antibiotic resistance. The latter was due to resistance gene duplication, an event rarely described in natural isolates. This is the first description of the *in vivo* occurrence of gene duplication in a mutator background.

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INTRODUCTION

Klebsiella pneumoniae is an important opportunistic pathogen responsible for a wide range of community and nosocomial infections, including urinary tract infections (UTIs), pneumonia, septicaemia and purulent abscesses (Podschun & Ullmann, 1998). A number of virulence factors contribute to the pathogenesis of this organism. However, the capsule is considered to be the dominant virulence factor, by mediating resistance to opsonization, phagocytosis and killing by fresh serum containing complement components (Podschun & Ullmann, 1998). In addition, this bacterial species has an intrinsic low-level resistance to amino- and carboxy-penicillins due to the

constitutive expression of a species-specific, narrow-spectrum, class A β -lactamase. Indeed, *K. pneumoniae* strains naturally possess a single chromosomal copy of a *bla*-encoding gene, *bla*_{SHV-1} or its derivative *bla*_{SHV-11}, or other closely related genes such as *bla*_{LEN} and *bla*_{OKP} (Chaves *et al.*, 2001; Haeggman *et al.*, 2004). High-level amoxicillin resistance can be due to mutations in the promoter region of these genes or to the acquisition of a plasmid-mediated β -lactamase (Arpin *et al.*, 2005; Rice *et al.*, 2000).

Since the end of the 1990s, strains with an unusually high spontaneous mutation rate, called mutators, have been observed in natural populations of pathogens (LeClerc *et al.*, 1996) and in the commensal flora (Matic *et al.*, 1997). Important levels of genetic variability (except when deleterious) confer selective advantages to bacteria in constantly changing environments (Taddei *et al.*, 1997; Denamur & Matic, 2006). Most mutator strains exhibit defects in the methyl-directed mismatch repair (MMR) system, the main post-replicative DNA repair pathway which protects against replication errors (Chopra *et al.*, 2003). In *Escherichia coli*, this system, directed by the Dam

Abbreviation: UTI, urinary tract infection.

The GenBank/EMBL/DDBJ accession numbers for the *mutS*, *mutH* and *mutL* sequences from Kp481 are DQ826449, DQ826450 and DQ826451, respectively, and of the 6.802 kb and 9.249 kb chromosomal *NcoI* fragments from Kp869 containing the *bla*_{SHV-11} β -lactamase gene are GQ463147 and GQ463148, respectively.

Two supplementary figures and a supplementary table are available with the online version of this paper.

methylase, involves three main proteins: MutS, MutL and MutH. MutS detects mispaired bases and recruits MutL, and the MutS/MutL complex activates the endonuclease MutH, which cleaves the newly synthesized, non-methylated daughter strand at its nearest hemi-methylated d(GATC) site. Then, strand removal and resynthesis steps are carried out by DNA-binding proteins, i.e. UvrD helicase, exonucleases and the DNA complex of replication (Schofield & Hsieh, 2003). In addition to the correction of DNA replication errors, the MMR proteins are potent inhibitors of recombination between non-identical DNA sequences (Junop *et al.*, 2003; Worth *et al.*, 1994). The mutator phenotype has been investigated in several bacterial species, and mutators have been found to occur at variable frequencies according to species and type of infection (Denamur *et al.*, 2002; LeClerc *et al.*, 1996; Oliver *et al.*, 2000; Prunier *et al.*, 2003; Watson *et al.*, 2004). Within the *Enterobacteriaceae*, the molecular basis of the mutator phenotype has been analysed in *E. coli* and *Salmonella enterica* (LeClerc *et al.*, 1996), but not in *K. pneumoniae*.

During a survey conducted in private health care centres in south-western France in 2004, a total of 110 non-redundant strains of *K. pneumoniae* were collected, mainly isolated from UTIs (63.6%) (Fischer *et al.*, 2004). The mutator phenotype prevalence was studied in this collection and a strain, Kp593, was found to exhibit a high rifampicin mutation frequency. The same mutator, Kp869, was collected 1 month later from the same patient. The molecular basis of the mutator phenotype was elucidated in both isolates, and additional phenotypic variations in Kp869, in particular the increased β -lactam resistance, were investigated.

METHODS

Bacterial strains and plasmids. The epidemiological relationship of two *K. pneumoniae* strains, Kp593 and Kp869, isolated from the same patient, was studied by using PFGE using *Xba*I-digested DNA and the CHEF-DRIII system according to the manufacturer's instructions (Bio-Rad). A non-mutator *K. pneumoniae* Kp481 was used as a control to determine rifampicin mutation frequencies, and as a source for the wild-type *mut* genes and the reference SHV-1 enzyme to determine β -lactamase content.

E. coli SM10 (Belgian Coordinated Collections of Microorganisms, Gent-Zwijnaarde, Belgium), which contains a part of the broad-spectrum conjugative plasmid RP4 integrated in its genome allowing mobilization of plasmids with an *oriT* transfer origin, was used as a donor strain in conjugation experiments with *K. pneumoniae* as recipient strains. *E. coli* JM109 (nalidixic acid resistant strain; Stratagene) was employed for cloning experiments and conjugation assays in β -lactam resistance analysis. Plasmid pEXTc18 was used in cloning experiments of *mut* genes and is a pUC18-derived plasmid with a tetracycline selectable marker and an *oriT* transfer origin for mobilization experiments (Hoang *et al.*, 1998). Plasmid pACYC184 (Stratagene) was used for *bla*_{SHV-11} gene cloning and is a pBR322-derived vector which also possesses a tetracycline resistance gene.

Antimicrobial susceptibility, mutation and reversion frequencies. Antibiotic susceptibility patterns of the strains were determined by the disc diffusion method in Mueller–Hinton (MH, Bio-Rad) agar

using 27 discs (Members of the SFM AntibioGram Committee, 2003). MICs of amoxicillin and ticarcillin (alone or in combination with 2 μ g clavulanic acid ml⁻¹), and cephalothin were determined by an agar dilution method in MH medium (Members of the SFM AntibioGram Committee, 2003).

To determine the mutation frequency of *K. pneumoniae* strains, cultures grown overnight at 37 °C in brain–heart infusion broth (Bio-Rad) were diluted, plated on MH agar medium with or without rifampicin at 200 μ g ml⁻¹, and then incubated for 24 h. Among the collection of 110 non-redundant strains of *K. pneumoniae*, the frequency of rifampicin-resistant mutants was between $<1 \times 10^{-9}$ and 6×10^{-8} , including a majority (56 strains) with a frequency ranging from $\leq 1 \times 10^{-9}$ to 5×10^{-9} , with a modal value of 5.0×10^{-9} , such as Kp481, which was taken as the non-mutator control strain. A single strain, Kp593, had a mutation frequency 100-fold superior to the modal value (Supplementary Fig. S1).

In order to estimate the stability of the high-level ampicillin resistance in Kp869, this strain was submitted to ten serial subcultures in antibiotic-free MH broth. The tenth subculture was diluted and plated on MH agar. The MIC of ampicillin was determined for 100 colonies taken at random. The reversion frequency was defined as the proportion of bacteria giving an MIC for ampicillin of 64 μ g ml⁻¹ instead of 1024 μ g ml⁻¹.

General DNA analysis procedures. Genomic and plasmid DNA was extracted using the protocols and reagents of commercial kits (genomic DNA and plasmid Midi kit, respectively; Qiagen). PCR amplifications were performed using *Taq* Gold polymerase (Applied Biosystems) and custom-made specific primers (Eurofins MWG Operon) (Supplementary Table S1). Sequencing was carried out with an automated fluorescent method based on the dye terminator chemistry (Ampli*Taq* DNA polymerase FS dye terminator cycle sequencing ready reaction kit, Applied Biosystems) and the ABI-3130xl sequencer (Applied Biosystems). For Southern blot analysis, labelling, hybridization and detection were performed using the DIG DNA kit according to the supplier's instructions (Roche Diagnostics). The probe for the *bla*_{SHV-11} gene consisted of a PCR product obtained from Kp869 with primers DownF3-SHV and OS13 (Supplementary Table S1).

***mut* gene cloning, complementation experiments and *mutL* site-directed mutagenesis experiments.** Based on the available genome sequence of *K. pneumoniae* MGH 78578 (GenBank/EMBL database accession no. NC_009648), oligonucleotides (Supplementary Table S1) containing restriction sites at their ends were designed to amplify the entire wild-type *mut* genes from Kp481, including their ribosome-binding sites (forward and reverse primers were, for *mutS*, MutSKpBH1 and MutSKpXb1; for *mutL*, MutLKpBH1 and MutLKpXb1; and for *mutH*, MutHKpE1 and MutHKpH 3, respectively). The amplicons of *mutS*, *mutL* and *mutH* were double-digested and ligated into the pEXTc18 cloning vector cut by the same enzymes, allowing their insertion in the same orientation as the *lacZ* promoter for optimal expression. *E. coli* JM109 was electrotransformed with the ligation mixtures, and cells were subsequently plated on MH agar containing tetracycline (15 μ g ml⁻¹), 5-bromo-4-chloro-3-indolyl- β -D-galactoside (40 μ g ml⁻¹) and isopropylthio- β -galactoside (40 μ g ml⁻¹). After analysis, the recombinant plasmids pEX-MutS, pEX-MutL and pEX-MutH were transferred into *E. coli* SM10. Mating experiments between the donor (*E. coli* SM10 containing the recombinant plasmids) and the recipient (*K. pneumoniae*) cells were performed by using a filter mating procedure (Courvalin *et al.*, 1985). Transconjugants selected on MH agar supplemented with tetracycline (15 μ g ml⁻¹) and ampicillin (30 μ g ml⁻¹) were obtained at a frequency of approximately 10^{-4} per donor. The site-directed mutagenesis of the *mutL* gene was performed using the plasmid pEX-MutL as DNA template, two pairs of complementary mutagenic oligonucleotide primers MutL307F and MutL307R (Supplementary

Table S1), and the QuikChange site-directed mutagenesis kit (Stratagene).

Negative staining of *K. pneumoniae* capsule. Cells from freshly grown colonies on MH medium were examined by transmission electron microscopy. In brief, bacterial ultrastructure Formvar-coated carbon-reinforced copper grid (200 mesh) was applied, film side down, on a droplet of a thick bacterial suspension on a paraffin strip. The grid was dried on filter paper and stained for 20 s on droplets of 2% ammonium molybdate, and excess liquid was sucked off with filter paper. Electron microscopy was carried out using a Hitachi H7650 electron microscope at 80 kV.

Analysis of β -lactamase content. The β -lactamase content of Kp593 and Kp869 was analysed by IEF after revelation by an iodine-starch procedure in an agar gel with benzylpenicillin ($75 \mu\text{g ml}^{-1}$) (Mathew *et al.*, 1975). Conjugation assays were carried out by using the filter-mating procedure using the nalidixic acid-resistant mutant of *E. coli* JM109 as recipient strain (Courvalin *et al.*, 1985). Plasmid DNA extracts were used for the electrotransformation of *E. coli* JM109. PCR amplification and sequencing of the entire *bla_{SHV}* gene (861 bp) and a 628 bp downstream region were carried out by using primers DownF3-SHV and OS13 (Supplementary Table S1).

***bla_{SHV-11}* cloning experiments and duplication analysis.** Cloning of the *bla_{SHV-11}* genes was performed by ligation of the *Nco*I-digested *K. pneumoniae* Kp869 genomic DNA and the *Nco*I-digested and alkaline phosphatase-treated plasmid pACYC184. The mixture was used to electrotransform the *E. coli* JM109 strain. Recombinant clones were selected on MH agar plates supplemented with tetracycline ($15 \mu\text{g ml}^{-1}$) and ampicillin ($50 \mu\text{g ml}^{-1}$). Based on the recently sequenced genome of *K. pneumoniae* 342 (Fouts *et al.*, 2008), a set of 11 primer pairs (Supplementary Table S1) was designed to generate overlapping PCR products in order to amplify the duplicated region.

Nucleotide sequence accession numbers. The *mutS*, *mutH* and *mutL* sequences from Kp481 have been deposited in GenBank under accession nos DQ826449, DQ826450 and DQ826451, respectively, and those of the 6.802 kb and 9.249 kb chromosomal *Nco*I fragments from Kp869 containing the *bla_{SHV-11}* β -lactamase gene have been deposited under accession nos GQ463147 and GQ463148, respectively.

RESULTS

Clinical and microbiological data

The patient carrying strain Kp593 developed a UTI caused by this strain on 21 February 2004. She was a 98-year-old

woman with multiple underlying diseases, residing in a nursing home. The UTI caused by this strain was successfully treated with norfloxacin ($2 \times 400 \text{ mg}$) for 5 days, but 1 month later (17 March 2004), the patient relapsed and *K. pneumoniae* strain Kp869 was recovered from a urinary sample. Unfortunately, the patient died 1 month later. Kp593 and Kp869 were clonally related as indicated by their *Xba*I DNA pulsotypes obtained after PFGE analysis (data not shown). The frequencies of rifampicin-resistant mutants were in the same order of magnitude for both Kp593 and Kp869, about 7.0×10^{-7} i.e. 100-fold higher than the modal value for the species (5.0×10^{-9}) (Table 1). However, both strains exhibited some phenotypic differences. Firstly, compared with Kp593, Kp869 gave more mucoid colonies on MH agar plates. A negative colouration followed by electron microscopy observation showed that the halo around the cells was thicker for Kp869 than for Kp593, suggesting that a greater amount of capsular polysaccharide was synthesized in Kp869 (Supplementary Fig. S2). Secondly, although by the disc diffusion method, Kp593 and Kp869 were susceptible to all antibiotics naturally active against *K. pneumoniae* species, their resistance patterns to amino- and carboxy-penicillins were different. Indeed, as verified by determination of MICs, the second isolate exhibited higher levels of amoxicillin and ticarcillin resistance, i.e. 1024 versus $64 \mu\text{g ml}^{-1}$ and 1024 versus $128 \mu\text{g ml}^{-1}$ for Kp869 and Kp593, respectively. Susceptibility was restored by the addition of clavulanic acid (amoxicillin-clavulanate, 1 and $4 \mu\text{g ml}^{-1}$, and ticarcillin-clavulanate, 2 and $8 \mu\text{g ml}^{-1}$, for Kp593 and Kp869, respectively). Both strains remained susceptible to cephalothin (MIC of $4 \mu\text{g ml}^{-1}$).

Molecular basis of the mutator phenotype in Kp593 and Kp869

Recombinant plasmids pEX-MutS, pEX-MutL or pEX-MutH containing the *mutS* (2.591 kb), *mutL* (2.037 kb) or *mutH* (0.898 kb) genes, respectively, were obtained from amplified genes of the non-mutator control strain, Kp481. The insert integrity was controlled by sequencing. By mating experiments, the wild-type *mutS*, *mutL* and *mutH* genes were transferred into Kp593, Kp869 and Kp481. The

Table 1. Frequencies of rifampicin-resistant mutants in *K. pneumoniae* strains

Means of at least three independent experiments ($\pm$ SD).

Native or recombinant plasmid	Mutator strain		Non-mutator strain Kp481
	Kp593	Kp869	
None	6.9×10^{-7} (± 1.9)	7.4×10^{-7} (± 3.2)	4.5×10^{-9} (± 1.9)
pEXTc18	5.6×10^{-7} (± 1.3)	8.0×10^{-7} (± 4.0)	4.8×10^{-9} (± 0.8)
pEX-MutS	7.3×10^{-7} (± 2.0)	5.2×10^{-7} (± 1.6)	3.9×10^{-9} (± 0.9)
pEX-MutH	6.1×10^{-7} (± 3.1)	4.8×10^{-7} (± 0.15)	2.8×10^{-9} (± 1.1)
pEX-MutL	4.1×10^{-9} (± 2.9)	3.9×10^{-9} (± 2.5)	4.5×10^{-9} (± 2.0)
pEX-MutLR307	1.7×10^{-7} (± 0.8)	1.2×10^{-7} (± 0.7)	1.6×10^{-7} (± 0.7)

rifampicin resistance frequencies were determined for each transconjugant (Tc). Results shown in Table 1 indicated that TcKp593 and TcKp869 were efficiently complemented by MutL, since the mutation frequency was reduced by a factor of 168–190, respectively. In contrast, no variations were observed after complementation with the wild-type MutS or MutH.

The *mutL* genes of Kp593 and Kp869 were sequenced and compared with those of the non-mutator, Kp481. The deduced MutL proteins of Kp593 and Kp869 were identical and harboured six amino acid substitutions (A201G, K307R, P349G, T373A, G388A and K389R) compared with MutL of Kp481. Among these mutations, only Lys307, located in the N-terminal conserved region of MutL, has been described as playing a key role in the *E. coli* MutL activity (Ban & Yang, 1998; Ban *et al.*, 1999). In order to confirm that the change K307R was responsible for the mutator phenotype in Kp593 and Kp869, this modification was generated by site-directed mutagenesis in *mutL* of pEX-MutL (MutL of Kp481). After a control (sequencing pEX-MutLR307) and complementation tests in the *K. pneumoniae* strains were carried out, the frequency of rifampicin-resistant mutants was determined, which showed that pEX-MutLR307 produced no significant changes in Kp593 and Kp869 (Table 1). These data demonstrated that the mutation Arg307 in MutL was the cause of the mutator phenotype in Kp593 and Kp869. In addition, pEX-MutLR307 increased the rifampicin resistance frequency of the wild-type strain Kp481 by approximately 300 times (Table 1).

Analysis of the *bla*_{SHV-11} duplication in Kp869

IEF analysis of the β -lactamase content of Kp593 and Kp869 revealed the presence of a single band with a pI of 7.6, co-focusing with the reference SHV-1 and SHV-11 enzymes, both differing by the single substitution Leu35Gln. β -Lactam resistance transfer by filter mating technique failed to yield any transconjugants. Plasmid DNA analysis did not reveal any plasmid in either strain. Selection of transformants on agar plates containing ampicillin after electrotransformation of *E. coli* by putative plasmid DNA extracts remained unsuccessful. Thus, these results strongly suggested that no β -lactamase-carrying plasmids were present in Kp593 or Kp869. PCR amplification and subsequent sequencing showed that the same *bla*_{SHV-11} gene was present in both strains, and that there was an identical sequence upstream from the *bla*_{SHV-11} gene, including the promoter region (Rice *et al.*, 2000). Furthermore, Southern blot hybridization analysis using the *bla*_{SHV-11} gene as a probe, performed on total unrestricted DNA, also indicated that this gene is chromosomally located, since only the genomic DNA positively hybridized with the probe without any additional band (data not shown).

After digestion of the DNA with the *Nco*I enzyme, which has no restriction site in the *bla*_{SHV-11} gene, Southern blot

experiments revealed that Kp869 chromosomal DNA had two *Nco*I fragments (6.8 and 9.2 kb), while a single *Nco*I fragment (6.8 kb) was present in Kp593 (Fig. 1). Identical results were obtained with *Eco*RI and *Hind*III (data not shown). The presence of this additional fragment indicated that the *bla*_{SHV-11} gene was duplicated in Kp869. To confirm this, *Nco*I fragments of whole-cell DNA of Kp869 were cloned in *E. coli* and the two recombinant plasmids pC1 and pC2, containing fragments of 6.802 kb and 9.249 kb, respectively, were obtained and the inserts were sequenced (Fig. 2a). The 3181 bp region upstream from the *bla*_{SHV-11} gene encompassing a part of the *lacZ* gene (encoding the enzyme for lactose catabolism), the *bla*_{SHV-11} sequence (861 bp) and the 827 bp region downstream from *bla*_{SHV-11}, were strictly identical in both *Nco*I fragments in pC1 and pC2. In contrast, the regions situated downstream were different (Fig. 2a). In pC2, the corresponding region fell into the beginning of the *yneI* gene encoding a putative NAD-dependent aldehyde dehydrogenase family protein. Amplification of Kp869 with primer pairs 1 and 2 (Fig. 2a) gave positive results, but did not with Kp593. Based on the use of a set of primer pairs, overlapping amplicons were obtained which allowed the distance between the 3' end (primer 3) and the 5' end in the duplicated region (primer 2) of the *yneI* gene to be estimated as 36.4 kb. Altogether, these results indicate the presence of a tandem duplication in Kp869 (Fig. 2a). It is noteworthy that, although the sequence analysis of both extremities of the amplified region did not reveal any significant sequence similarity, except for the repetition of

Fig. 1. *bla*_{SHV-11} hybridization of *Nco*I-digested Kp593 and Kp869 DNA. M, Labelled lambda phage DNA digested by *Pst*I, used as size marker.

Fig. 2. Schematic representation of *bla*_{SHV-11} gene and surrounding DNA in Kp593 and Kp869 strains. (a) The 6.8 and 9.2 kb *Nco*I fragments are represented. Genes are indicated by boxes and their transcriptional orientations are indicated by arrows. These were defined according to homologies found in GenBank databases, and their putative functions are further described in the records with GenBank accession nos GQ463147 (6.8 kb fragment) and GQ463148 (9.2 kb fragment). The gene boxes are as follows: black fill, *bla*_{SHV-11}; cross-hatched fill, unique genes in the 6.8 kb *Nco*I-fragment; vertical and horizontal lines, 3' and 5' duplicated ends of the *yneI* gene, respectively; dark and light grey fill, *deoR1* and *deoR2* genes, respectively. The small arrows indicate primer binding sites and their orientation. (b) Nucleotide sequences of the junction site and of left and right extremities are indicated. The repeated dinucleotide TA is boxed.

the dinucleotide TA (Fig. 2b), two genes, *deoR1* and *deoR2* exhibiting 46 % homology, were present in the duplicated region (Fig. 2a)

Instability of the *bla*_{SHV-11} duplication in Kp869

In order to estimate the stability of this duplication, ten serial passages of Kp869 were performed in an antibiotic-free medium. At the last subculture, 12 of the 100 tested clones had MICs of ampicillin of 64 µg ml⁻¹, similar to Kp593, instead of 1024 µg ml⁻¹, as for Kp869. Amplifications with the primer pairs 1 and 2 for these 12 clones remained negative, suggesting that they lost the tandem duplication. Nevertheless, these 12 clones remained mutators, with high mutation frequencies to rifampicin resistance (data not shown).

DISCUSSION

The prevalence of the mutator phenotype in a *K. pneumoniae* collection was found to be 1 %. Similar rates were observed in natural populations of *E. coli* and *Salmonella* (Galán *et al.*, 2004; LeClerc *et al.*, 1996). However, hypermutable strains have also been described in Gram-negative bacteria at variable frequencies depending on not only the bacterial species or the phylogenetic group of strains but also their ecosystem (Denamur *et al.*, 2002; Oliver *et al.*, 2000). To our knowledge, a single study has

mentioned mutation frequencies in clinical *K. pneumoniae* isolates, but their data on mutation rates were difficult to use for comparison due to methodological differences (De Champs *et al.*, 2004).

Until now, no molecular experiments have elucidated the mutator phenotype in *K. pneumoniae* to our knowledge. Alterations in MutS have been predominantly described as being responsible for the phenotype in *E. coli* clinical strains (LeClerc *et al.*, 1996), and they usually lead to a 100-fold increase in the mutation rate, i.e. close to the value obtained for the Kp593 strain (LeClerc *et al.*, 1996). Nevertheless, our complementation data demonstrated that MutL was involved in the mutator phenotype of Kp593. MutL of *E. coli* is a homodimeric protein of 615 amino acids (619 residues in *K. pneumoniae*). It contains a conserved N-terminal ATPase domain (LN40) ranging from amino acids 1 to 330, a divergent C-terminal dimerization region (LC20, residues 430–615) and a 100 residue proline-rich linker which generates a large central cavity and tolerates sequence substitutions and deletions of one-third of its length without any functional consequence *in vivo* and *in vitro* (Ban & Yang, 1998; Ban *et al.*, 1999; Guarné *et al.*, 2004). Most amino acid differences in MutL between the non-mutator (Kp481) and the mutator (Kp593 and Kp869) strains were located in the variable linker region (Ban & Yang, 1998). A single one corresponded to a highly conserved position in the N-terminal, i.e. the 307 position (Ban & Yang, 1998). The change

Lys310Met (equivalent to Lys307 in MutL of *E. coli*) has been suspected to be associated with the mutator phenotype in a clinical strain of *Pseudomonas aeruginosa* (Oliver *et al.*, 2002). Another study has shown that *in vitro* mutagenesis experiments in *E. coli*, changing Lys307 to Ala, yielded an enzyme with a drastic decrease in ATP hydrolysis (Ban & Yang, 1998; Junop *et al.*, 2003). However, the replacement of the basic amino acid lysine by another basic residue arginine in Kp593/Kp869 might have been less disturbing for the protein than its substitution by methionine or alanine. In fact, the introduction of the defective protein MutL-R307 had no effect in the mutators, confirming the role of this residue in the mutator phenotype. In contrast, the introduction of MutL-R307 from a multicopy plasmid in the wild-type strain Kp481 resulted in an important increase in the mutation frequency. Such a dominant-negative effect of a MutL mutant in a wild-type host cell background might correspond to the formation of inactive heteromultimers composed of wild-type and mutant MutL monomers (Aronshtam & Marinus, 1996).

Bacterial cells with a high mutation rate have a selective advantage because they adapt faster than non-mutators (Denamur & Matic, 2006). Thus, in our study, the thickness of the outer layer of Kp869 was predicted on the basis of the appearance of the colonies which were more mucoid, together with a larger halo visualized by electron microscopy. Capsule production is influenced by mutations in the loci encoding capsular polysaccharides and their regulatory regions (Lai *et al.*, 2003), and has been linked to virulence in animal models (Wu *et al.*, 2009) and clinical observations (Yu *et al.*, 2007). Higher levels of capsule production might have contributed to an increase in virulence of the mutator.

Mutations that confer a selective advantage can increase virulence and also other bacterial defence systems such as antibiotic resistance (Denamur & Matic, 2006). In our study, the increased β -lactam resistance in Kp869 was due to a duplication event resulting in a gene dosage effect, although the patient received a fluoroquinolone instead of a β -lactam antibiotic. However, a non-documented previous treatment with β -lactams in this 98-year-old patient residing in a nursing home cannot be totally excluded. Furthermore, a selection pressure acting on another gene present in the duplicated region is also possible. In this instance, duplications of the *lac* operon, associated with faster growth on lactose, have been reported (Lupski *et al.*, 1996). In clinical *K. pneumoniae* strains, *bla*_{SHV} gene amplifications have been previously described but they corresponded to duplications associated with the presence of mobile genetic elements such as the IS26 insertion sequence (Hammond *et al.*, 2008; Sandegren & Andersson, 2009). Another study reported that 8.2 % of *K. pneumoniae* strains possess a locus containing both *bla*_{SHV-1} and *bla*_{LEN-1} (the coded enzymes sharing 90.9 % homology), possibly arranged in tandem but without further confirmation (Chaves *et al.*, 2001). In addition,

analysis of the recently sequenced *K. pneumoniae* 342 genome revealed that the *bla*_{SHV-11} gene was located on a 16 kb duplicated fragment (Fouts *et al.*, 2008). Although duplications of bacterial chromosomal regions are known to arise at high frequencies under laboratory conditions (Romero & Palacios, 1997), the demonstration of such resistance gene amplification, with analysis of the initial isolate lacking any duplication and the isogenic one with an acquired duplication, has been rarely documented in natural isolates and this has not included analysis of the mutator capacity of the strains (Brochet *et al.*, 2008). The generated long direct repeated DNA regions are unstable, and our *in vitro* experiments confirmed the instability of this gene amplification. The low number of reported cases probably reflects the transient nature of gene amplification (Andersson & Hughes, 2009; Sandegren & Andersson, 2009). A link between mutator phenotype and gene duplication might exist. Indeed, in the literature, gene amplifications can occur by either RecA-dependent or -independent mechanisms (Andersson & Hughes, 2009; Sandegren & Andersson, 2009). In the first case, the recombination fidelity in the strands exchange process is controlled by MutS and MutL, which recognize mispaired and unpaired bases in the joint heteroduplex regions, and subsequently block RecA-catalysed strand transfer (Worth *et al.*, 1994). Accordingly, a deficient MutL could favour the recombination of not completely homologous strands, i.e. between *deoR* sequences.

In conclusion, the mutator frequency in a natural population of *K. pneumoniae* was around 1 %. This is the first elucidation, to our knowledge, of the molecular basis of the mutator phenotype in this species. The presence of the mutator phenotype in this clinical strain has probably conferred a selective advantage, such as increased capsule production and/or antibiotic resistance. In this case, increased ampicillin resistance was linked to the gene duplication, a genetic event rarely reported in natural isolates. Our study is the first description to our knowledge of the presence of both events, i.e. mutator phenotype and gene duplication, in a clinical strain and demonstrates the great adaptability of *K. pneumoniae* strains to their environment. The reversion of wild-type phenotype and the instability of DNA segment amplifications without selection pressure suggest that underlying genetic modifications could play an important, currently underestimated, role in the emergence of antibiotic resistance.

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