A 4.5-Year Within-Patient Evolution of a Colistin-Resistant Klebsiella pneumoniae Carbapenemase-Producing K. pneumoniae Sequence Type 258

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Agnès B. Jousset[1,2,3,4], Rémy A. Bonnin[2,3,4], Isabelle Rosinski-Chupin[4,5], Delphine Girlich[3,4], Gaëlle Cuzon[1,2,3,4], Nicolas Cabanel[4,5], Hélène Frech[6], Eric Farfour[7], Laurent Dortet[1,2,3,4], Philippe Glaser[4,5*], and Thierry Naas[1,2,3,4*]

[1] Department of Bacteriology-Parasitology-Hygiene, Hôpital de Bicêtre, Assistance Publique - Hôpitaux de Paris, Le Kremlin-Bicêtre, France

[2] Associated French National Reference Center for Antibiotic Resistance, Le Kremlin-Bicêtre, France

[3] EA7361 "Structure, dynamic, function and expression of broad spectrum β-lactamases", Paris-Sud University, Faculty of Medicine, Le Kremlin-Bicêtre, France

[4] Joint research Unit EERA « Evolution and Ecology of Resistance to Antibiotics », Institut Pasteur-APHP-University Paris Sud, Paris, France

[5] CNRS, UMR 3525, Paris, France

[6] Department of Biology, Hôpital de Poissy–Saint-Germain-en-Laye, Poissy, France

[7] Department of microbiology, Hôpital Foch, Suresnes, France

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* Corresponding authors:

Philippe Glaser, Unité EERA, Institut Pasteur, 28 Rue du Dr Roux, 75724 Paris, Cedex 15, Phone: +33 1 45 68 89 96. e-mail: philippe.glaser@pasteur.fr

Thierry Naas, Service de Bactériologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre, France. Phone: +33 1 45 21 29 86. e-mail: thierry.naas@aphp.fr

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40-word summary:

KPC-producing *Klebsiella pneumoniae* (KPC-Kp) have emerged as a major nosocomial pathogen. This study emphasizes long-term *in-vivo* genome evolution along with the selection of pathoadaptive mutations in a KPC-Kp strain that might have contributed to its long-term carriage, virulence and dissemination.

ABSTRACT

Background. KPC-producing *Klebsiella pneumoniae* (KPC-Kp) have emerged globally over the last decade as a major nosocomial pathogen that threatens patient's care. These highly resistant bacteria are mostly associated with a single Kp clonal group (CG) CG258, but the reasons for its host and hospital adaptation remain largely unknown.

Methods. We analyzed the *in vivo* evolution of a colistin-resistant KPC-Kp-CG258 strain that contaminated a patient following an endoscopy, and was responsible for a fatal bacteraemia 4.5 years later. Whole Genome Sequencing (WGS) was performed on 17 KPC-Kp isolates from this patient, single-nucleotide polymorphisms (SNPs) were analyzed and their implication in antimicrobial resistance and bacterial host adaptation investigated.

Results. The patient KPC-Kp strain diversified over 4.5 years at a rate of 7.5 substitutions per genome per year resulting in broad phenotypic modifications. After two years of carriage, all isolates restored susceptibility to colistin. Higher expression of the fimbriae conferred the ability to produce more biofilm and the isolate responsible for a bacteraemia grew in human serum. The convergent mutations occurring in specific pathways, such as the respiratory chain and the cell envelope revealed a complex long-term adaptation of KPC-Kp.

Conclusion. Broad genomic and phenotypic diversification and the parallel selection of pathoadaptive mutations might contribute to long-term carriage and virulence of KPC-Kp-CG258 strains and to the dissemination of this clone.

The increasing incidence of extended spectrum β-lactamase (ESBL) and carbapenemase-producing strains of *K. pneumoniae* (Kp) in health care facilities is a cause of global concern. In several countries including Greece, Italy, Israel and the United States, the rate of infections with *K. pneumoniae* resistant to all antibiotics commercially available is worryingly growing [1–5]. Most of these isolates are resistant to all β-lactams due to the production of a class A carbapenemase named KPC and can also be resistant to other antibiotics of last resort like tigecylin [6] and colistin [7]. The worldwide dissemination of KPC-Kp is mainly associated with the spread of a single clonal group designated CG258 [1,8,9].The evolutionary success of CG258 in combination with KPC is not yet fully understood. Several factors might have an impact on the global dissemination of a clone, such as increased pathogenicity, high transmissibility, and/or increased duration of colonization [9]. Analysis of KPC-Kp ST258 outbreaks indicated that this clone can be easily transmitted between patients for several months following the identification of the initial index case [8]. However, little is known about the outcome of KPC-Kp fecal carriers and the duration of carriage, which are determining transmission factors. Some studies indicate that patients can exhibit spontaneous decolonization within 6 months after KPC-Kp acquisition [10]. In contrast, reports of carriage longer than one year has been also observed [8,10,11].

Here we analyzed the evolution of a KPC-Kp ST258 strain that colonized a patient over 4.5 years. This strain underwent considerable genetic and phenotypic diversification to adapt to antibiotic treatments and to promote virulence. These results highlight the capacity of adaption of a KPC-Kp ST258 strain that may contribute to its propensity to sustainably colonize patients and persist in healthcare institutions.

MATERIALS AND METHODS

Accession numbers

Kp BIC-1 sequences have been submitted to Genbank under the accessions numbers: CP022573 (chromosome), CP022574 (pBIC1-a), CP022575 (pBIC-1b), CP022576 (pBIC-1c).

Bacterial strains

Strain Kp BIC-1 transmitted to the patient was recovered from a contaminated endoscope [12,13]. Strains S1 to S17 were isolated from different body sites and are summarized in Table 1. Kp CIP 53153, YC2004 (KPC-Kp ST258) [14] and KN633 (KPC-Kp ST338) [14] are isolates susceptible to colistin used as control in qRT-PCR experiments. Kp LM21 was used as positive control in biofilm formation [15]. Electrocompetent *Escherichia coli* TOP10 (ThermoFisher, Villebon Sur Yvette, France) was used as a recipient in electroporation experiments.

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) for carbapenems (imipenem, ertapenem and meropenem), aminoglycosides (gentamicin, amikacin, tobramycin and netilmicin) and tigecyclin were determined using Etest® strips (bioMérieux) on Mueller-Hinton agar plates (Bio-Rad). The MICs for colistin were determined by broth culture microdilution in triplicate. Results were interpreted according to the EUCAST guidelines (www.eucast.org).

Whole-genome sequencing (WGS) and bioinformatics analysis

The genome of Kp BIC-1 was sequenced using PacBio RS technology (GATC Biotech AG, Mulhouse, France). Illumina WGS was performed on Kp isolates BIC-1 and S1 to 17. DNA libraries were prepared using the Nextera XT v3 kit (Illumina, Essex, UK) and run on a MiSeq system to generate paired-end 75- or 150-bp reads. Kp BIC-1 genome was used as

reference for detecting mutations using BRESEQ [16]. Acquisition of genetic material was assessed by analyzing unmapped reads following their *de novo* assembly by using Velvet [17]. Phylogeny of the 18 Kp isolates was constructed by considering the 85 identified SNPs among their genomes by Maximum-Parsimony (MP) using MEGA [18], refined manually and rooted by considering the direction of each SNP as described previously [19]. Evolutionary rate of the patient strain was determined based on the linear association between the root-to-tip number of mutations and year of strain isolation. The core genome phylogeny of the 18 Kp isolates together with ST258 published sequences and with the ST11 strain HS11286 as an outgroup was performed using the Parsnp program from the Harvest suite [20].

Quantification of biofilm formation

Biofilm formation capacity was measured in microtiter plate as previously described [21]. Briefly 2.10^7 CFU/mL of an overnight culture were inoculated into 180 µl of medium M63B1–0.4% Glucose in a 96-well PVC microtiter plate (Falcon). After 18h of incubation at 37°C, culture medium was removed, bacteria were fixed by adding 100 µl of Boin's solution (Sigma-Aldrich, Saint Quentin Fallavier, France) and rinsed once with distilled water. Bacterial cells were stained for 10 min at room temperature by adding 50 µl of 0.5% (wt/vol) aqueous solution of crystal violet in each well and washed twice with distilled water and dried at room temperature. The bound dye was released from stained cells using 80/20 ethanol/acetone solution and measured by absorbance at 595 nm.

Serum resistance assay

The serum resistance of isolates S1, S11, S13, S15, S17 and Kp CIP53153 as reference were determined according to the method of Siu *et al* [22]. Briefly, bacterial suspensions were collected from mid-log phase cultures (approximately 5.10^7 CFU/ml) and mixed at a 1:3

(vol/vol) ratios with pooled human serum (Sterile and filtered human male AB plasma from Sigma-Aldrich) and incubated at 37°C with agitation. Colony counts were determined at T0 and after 30, 60, 120 and 180 min of incubation. Serum susceptibility was assessed by plotting the average survival percentage against the incubation time. Each strain was tested four times.

Statistical analysis data

Statistical analysis was performed on ≥ 3 independent experiments using GraphPad Prism v6 software. Data were compared two-by-two using unpaired Student's t test with Welch'correction. p values <0.05 were considered statistically significant.

RESULTS

Clinical case

In 2009, a nosocomial outbreak of KPC-Kp isolates mediated by a contaminated endoscope occurred in a French tertiary care hospital [12]. Seventeen patients that underwent gastroscopy were contaminated with an endoscope-borne KPC-Kp strain (Kp BIC-1). One of them suffered from recurrent cholangitis due to repetitive lithiasis (Figure 1A). In September 2009, he underwent sphincteroscopy with the contaminated endoscope. Until April 2014, 17 KPC-Kp isolates were collected from this patient (S1 to S17) (Table 1). In 2013, a prostatic adenocarcinoma and a bladder cancer were diagnosed. In 2014, infectious complications occurred a week after a cystoprostatectomy, with peritonitis secondary to a breach in the Bricker anastomosis. Sepsis secondary to a polymicrobial infection with intestinal bacteria including KPC-Kp was followed by multivisceral failure and death.

Genetic diversification of a single KPC-Kp strain

To demonstrate whether the patient was colonized by a single KPC-Kp strain, we performed Illumina WGS of the 17 isolates and determined the complete genome sequence of Kp BIC-1 strain by combining PacBio and Illumina sequencing data. Kp BIC-1 carries a circular chromosome of 5,394,314 bp containing 5,189 predicted coding sequences and three plasmids: pBIC-1a (a 170,415 bp IncFIIK2 family, pBIC-1b (a 43,380 bp IncX3 plasmid) and pBIC-1c (a 13,841 bp ColE1 plasmid). Multiple antimicrobial, heavy-metal and arsenic resistance markers were carried by the chromosome and the three plasmids (Supplementary Table 1). Phylogenetic analysis with representative KPC-Kp ST258 isolates showed that this strain belongs to clade I of ST258 (Supplementary Figure 1) [2,23,24]. Single nucleotide polymorphisms (SNPs) based phylogeny of the 18 isolates showed that they represent a monophyletic clade (Figure 1B). Therefore, all the patients' isolates derived from Kp BIC-1.

Within-host colonization of this strain over 4.5 years led to the accumulation of mutations (deletions, tandem duplications and SNPs) in up to 39, 24 and 22 loci for the last three isolates S15, S16 and S17, respectively (Figure 1B). Assembly of unmapped reads revealed no gene acquisition and a remarkably stable plasmid content over 4.5 years of carriage. A total of 98 genetic events occurred (Supplementary Table 2), with most of them in the chromosome (n=91). From this longitudinal study, we estimated that the average evolutionary rate of this KPC strain was of 7.5 SNPs per year per genome (Figure 1C).

Most significantly, the functions affected showed convergence towards specific pathways (Supplementary Table 2): (i) the respiratory chain: with three independent mutations in complex 1, heme biosynthesis and in the *sucC* gene, (ii) anaerobic respiration with mutations in nitrate reductase genes, in the promoter region of the *fnr* gene encoding a regulator of anaerobic metabolism and in the *narL* regulatory gene, (iii) the metabolism of different carbon sources and (iv) the cell envelope with mutations in porin genes (two independent mutations in the ferric enterobactin receptor precursor, *pfeA* and an *ompF*-like

187 gene), capsular polysaccharides (the glycosyl transferase *epsJ*, the colanic acid biosynthesis
188 gene *wcaJ* and the sensor histidine kinase *rcsC*) and the *rfaH* antiterminator.
189
190 **Restoring colistin susceptibility**
191 The patient received during these 4.5 years numerous antimicrobial treatments,
192 including carbapenems and gentamicin (Figure 1A). Accordingly, we observed changes in the
193 antibiotic susceptibility during colonization (Table 1). Kp BIC-1 isolated from the endoscope
194 and the first twelve isolates from the patient (S1 to S12) were resistant to colistin with MICs ≥
195 64 mg/L (Table 1). This high level of resistance resulted from the insertion of IS*Kpn*25
196 between nucleotides 141 and 142 of the *mgrB* gene. MgrB plays a prominent role in colistin
197 resistance in Kp by repressing the PhoP-PhoQ regulatory pathway [25,26]. PhoP indirectly
198 activates the PmrA-PmrB two component system, which results in the overexpression of the
199 *arnBCADTEF* operon leading to addition of the cationic groups (4-amino-4-deoxy-L-
200 arabinose, L-Ara4N) on the phosphate groups of lipid A. This modification results in a
201 decrease of the electronegative charge of the bacterial surface and resistance to cationic
202 peptides [27]. After two years of carriage without any colistin treatment, all isolates (S13 to
203 S17) were susceptible to colistin (Table 1). We identified two mutations in two partners of the
204 colistin resistance pathway that may explain this phenotypic reversion. In four isolates (S13,
205 S14, S16 and S17) we identified a mutation in *arnC*, leading to a non-conservative amino-
206 acid change C161Y. ArnC is a lipid-A glycosyltransferase encoded by the *arnBCADTEF*
207 operon. Isolate S15 is mutated in *phoP* (I201N). To investigate the effect of this SNP in PhoP,
208 we quantified the expression of *arnC*, *phoP* and *phoQ* genes (indirect and direct targets of
209 PhoP) by qRT-PCR on strain S1 resistant to colistin, S15 (PhoP I201N), S17 (ArnC C161Y)
210 and on three colistin susceptible Kp strains used as references (CIP 53153, YC2004, KN533).
211 Compared to the reference strains, isolates S1 and S17 showed a significantly higher

expression of *arnC*, confirming the effect of the *mgrB* disruption (Supplementary Figure 2). In the S15 isolate mutated in PhoP, the expression of *arnC, phoP* and *phoQ* was restored at the level of the reference colistin susceptible strains (Supplementary Figure 2). Regarding the S17 isolate, no change in the expression of *arnC, phoP* and *phoQ* was observed as compared to S1. We therefore hypothesized that the mutation in *arnC* led to a decrease of the glycosyltransferase activity of the modified ArnC protein. To confirm the role of the SNPs in *phoP* and *arnC* in colistin susceptibility reversion, we complemented the phenotype of S15 and S17 respectively by cloning the unmutated gene into a modified pUC19 vector (Supplementary methods). Both complemented isolates S15-pUC19-*phoP*(WT) and S17-pUC19-*arnC*(WT) were resistant to colistin with MICs ≥ 32 µg/ml (Table 2) .

Variation of aminoglycoside susceptibility

Kp-BIC-1 and the first six isolates were susceptible to gentamicin whereas six later isolates (S7 to S11 and S15) shifted to gentamicin resistance and an increased susceptibility to amikacin (Table 1 and Supplementary Table 3). SNPs analysis revealed the presence of a point mutation in *aac*(6')-Ib gene carried by pBIC-1c leading to a L119S amino-acid change of the AAC(6')-Ib enzyme in all the gentamicin resistant isolates. Casin et *al.* showed that this substitution led similarly to an 8-fold decrease in amikacin MICs and to an 8-fold increase in gentamicin MICs [28]. By cloning the two *aac*(6')-Ib variants in *E. coli,* the involvement of this SNP for the gentamicin resistance switch was demonstrated (Supplementary Table 3).

Strain adaptation to promote virulence

Specific adaptation that could promote bacterial virulence during long-term carriage was investigated. A feature of KPC-Kp ST258 isolates is their ability to cause bacteraemia especially in immunocompromised patients [29]. Among the 17 isolates, S15 was responsible

for a bloodstream infection. To assess if some polymorphisms in S15 might have contributed to its ability to grow in blood, we performed serum resistance assay on five isolates from the patient and on the non-ST258 Kp CIP 53153 strain as reference. Isolates S1, S11, S13, S17 and CIP 53153 were highly sensitive to serum but isolate S15 was not only resistant to human serum but also grew rapidly (Figure 2). An SNP responsible for a nonsense mutation was present in the *gmlC* gene only in this isolate. The *gml* operon is present in the vast majority of KPC-Kp ST258 strains and is known to confer improved survival in the presence of human serum [30]. However, cloning experiment of a wild-type copy of *gmlC* in S15 could not restore susceptibly to human serum (Supplementary methods). The reasons of S15 peculiar phenotype might reside in one or several of the other 35 unique mutations.

A second common feature of KPC-Kp ST258 is their low capacity to produce biofilm [31]. However mutations in type 1 fimbriae operon potentially implicated in biofilm production [32] were detected in several isolates. Indeed, whereas the Kp-BIC-1 strain and 16 isolates were poor biofilm producers, the isolate S7 produced significantly more biofilm (Figure 3A). We have detected three differences in the genome of S7 compared to Kp-BIC-1 (Supplementary Table 2) including an inversion of the *fim* element in the promoter region switching from off to on the expression of the type 1 fimbriae operon (Figure 1C) and a non-synonymous SNP (P180T) in the *fimH* gene encoding the fimbriae adhesin.

Isolates S6, S8, S9, S10, S15 and S17 possess also an insertion of an adenosine in the *fim* element (Figure 3B) but no significant difference in biofilm formation (Figure 3A). To investigate the effect of these genomic modifications on the expression of the type 1 fimbriae, we performed qRT-PCR on isolates Kp BIC-1, S6 and S7 (Supplementary Materials). Compared to Kp BIC-1, we observed 11- and 71-fold increase in the expression of the *fimH* gene in S6 and in S7 respectively (Figure 3C). Although the 11-fold increase in *fimH*

expression has no detectable effect on biofilm production under laboratory conditions, it might increase adhesion to host cells and contribute to virulence or colonization [33].

DISCUSSION

Here, we deciphered by WGS the within patient evolution of a single strain of KPC-Kp over 4.5 years. The study begins with a rare but yet dramatic case of nosocomial transmission of carbapenemase-producing Enterobacteriaceae due to a contaminated endoscope, and ends prematurely with the patient's death secondary to a sepsis with the same KPC-Kp. The evolutionary rate was calculated at 7.5 SNPs per year per genome but this value needs to be considered with caution since the patient received several courses of antimicrobial treatments and thus, the selective pressure was not constant. This value is between the rate of 3.8 SNPs/year observed in a study of MDR Kp in Ireland and UK [34] and a rate of ten mutations per genome per year determined in KPC-Kp from a single institution [35].

We therefore analyzed the phenotypic diversification related to antimicrobial pressure. Regarding aminoglycosides susceptibility, we reported a *in vivo* mutation in the *aac*(6')-Ib gene that conferred resistance to gentamicin. It appeared subsequent to a ten days treatment with imipenem and gentamicin, and was stable over-time. This highlights the risk of rapid change in the specificity of AAC(6')-Ib and its possible implication in treatment failures. Strikingly, the contaminating strain was colistin resistant but after two years of carriage, all isolates became susceptible to colistin. The *in vivo* reversion from a resistant to a susceptible phenotype has been reported in *Acinetobacter baumannii* and in *Pseudomonas aeruginosa* but not yet in Kp [27]. Colistin are currently one of the last resort treatment for severe human infections caused by MDR bacteria, particularly carbapenemase-producing Enterobacteriaceae [7]. Even though plasmid-mediated colistin resistance *mcr* genes have recently been described worldwide [36,37], high level colistin resistance in

Enterobacteriaceae in human is still mainly due to non-transferable modifications of the lipid A resulting from chromosomal mutations [27]. After two years of intestinal carriage without colistin pressure, we identified two compensatory mutations in the clinical isolates, highlighting the potential impact of lipid A modifications on bacterial *in vivo* fitness, similarly to what was observed for colistin-resistant *A. baumannii* both *in vitro* and *in vivo* [38].

To further explore the long-term adaptation of this clone, mutations that could impact bacterial virulence were searched for. An inversion in the promoter region of the *fim* locus was found in isolate S7 capable to produce significantly more biofilm than others. Search for the orientation of the *fim* element in KPC-Kp CG258 genome sequences available on public repositories revealed that out of more than 500 strains, only three were oriented to allow expression of the *fim* operon. Therefore, expression of the type 1 fimbriae seems extremely rare in KPC-Kp ST258 and might have been selected under long-term colonization. Furthermore, isolate S15 showed a unique evolutionary trajectory in the patient, as it did not share any common SNPs with the previous nor the two following isolates and had 39 genomic differences with BIC-1 (Figure 1B and Supplementary Table 2). This isolate is therefore a proof of the genomic diversity that occurred *in vivo* during persistent carriage leading to a broad range of adaptive phenotypes contributing to invasive infection. S15 strain isolated almost three years after infection acquired the ability to grow in serum, to resist to gentamicin and reversed the colistin resistance phenotype. The diversity of isolates revealed a complex within host evolution involving possibly adaptation to specific niches in the gut and leading to a diverse clonal population only partially characterized by the conservation of a single isolate at each sampling.

In summary, our work highlights the importance of leveraging the power of WGS to better understand how clinically-important clones are capable to adapt and cause disease in the human host. Currently, no specific clinical guidelines recommend the typing of more than

311 one isolate per patient, but our findings reveal the need to closely consider within-host
312 variability in future studies of *K. pneumoniae* evolution and transmission.
313
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321 **Conflict of interest**
322 The authors declare no competing financial interests
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References

1. Pitout JDD, Nordmann P, Poirel L. Carbapenemase-Producing Klebsiella pneumoniae, a Key Pathogen Set for Global Nosocomial Dominance. Antimicrob Agents Chemother **2015**; 59:5873–5884.

2. Wright MS, Perez F, Brinkac L, et al. Population Structure of KPC-Producing Klebsiella pneumoniae Isolates from Midwestern U.S. Hospitals. Antimicrob Agents Chemother **2014**; 58:4961–4965.

3. Mavroidi A, Katsiari M, Likousi S, et al. Characterization of ST258 Colistin-Resistant, blaKPC-Producing Klebsiella pneumoniae in a Greek Hospital. Microb Drug Resist **2016**; 22.

4. Rojas LJ, Weinstock GM, De La Cadena E, et al. An Analysis of the Epidemic of Klebsiella pneumoniae Carbapenemase-Producing K. pneumoniae: Convergence of Two Evolutionary Mechanisms Creates the 'Perfect Storm'. J Infect Dis **2017**; 217:82–92.

5. Zowawi HM, Forde BM, Alfaresi M, et al. Stepwise evolution of pandrug-resistance in Klebsiella pneumoniae. Sci Rep **2015**; 5:15082.

6. He F, Fu Y, Chen Q, et al. Tigecycline Susceptibility and the Role of Efflux Pumps in Tigecycline Resistance in KPC-Producing Klebsiella pneumoniae. PLOS ONE **2015**; 10:e0119064.

7. Rojas LJ, Salim M, Cober E, et al. Colistin Resistance in Carbapenem-Resistant Klebsiella pneumoniae: Laboratory Detection and Impact on Mortality. Clin Infect Dis Off Publ Infect Dis Soc Am **2017**; 64:711–718.

8. Mathers AJ, Peirano G, Pitout JDD. The Role of Epidemic Resistance Plasmids and International High-Risk Clones in the Spread of Multidrug-Resistant Enterobacteriaceae. Clin Microbiol Rev **2015**; 28:565–591.

9. Dautzenberg MJD, Haverkate MR, Bonten MJM, Bootsma MCJ. Epidemic potential

351 of Escherichia coli ST131 and Klebsiella pneumoniae ST258: a systematic review and meta-
352 analysis. BMJ Open **2016**; 6:e009971.
353 10. Feldman N, Adler A, Molshatzki N, et al. Gastrointestinal colonization by KPC-
354 producing Klebsiella pneumoniae following hospital discharge: duration of carriage and risk
355 factors for persistent carriage. Clin Microbiol Infect **2013**; 19:E190–E196.
356 11. Conlan S, Park M, Deming C, et al. Plasmid Dynamics in KPC-Positive Klebsiella
357 pneumoniae during Long-Term Patient Colonization. mBio **2016**; 7:e00742-16.
358 12. Naas T, Cuzon G, Babics A, et al. Endoscopy-associated transmission of carbapenem-
359 resistant Klebsiella pneumoniae producing KPC-2 β-lactamase. J Antimicrob Chemother
360 **2010**; 65:1305–1306.
361 13. Dortet L, Naas T, Boytchev I, Fortineau N. Endoscopy-associated transmission of
362 carbapenemase-producing Enterobacteriaceae: return of 5 years' experience. Endoscopy
363 **2015**; 47:561–561.
364 14. Cuzon G, Naas T, Truong H, et al. Worldwide Diversity of *Klebsiella pneumoniae*
365 That Produce β-Lactamase *bla* $_{KPC-2}$ Gene1. Emerg Infect Dis **2010**; 16:1349–1356.
366 15. Favre-Bonte S, Joly B, Forestier C. Consequences of reduction of Klebsiella
367 pneumoniae capsule expression on interactions of this bacterium with epithelial cells. Infect
368 Immun **1999**; 67:554–561.
369 16. Deatherage DE, Barrick JE. Identification of mutations in laboratory evolved microbes
370 from next-generation sequencing data using breseq. Methods Mol Biol Clifton NJ **2014**;
371 1151:165–188.
372 17. Zerbino DR, Birney E. Velvet: Algorithms for de novo short read assembly using de
373 Bruijn graphs. Genome Res **2008**; 18:821–829.
374 18. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis
375 Version 7.0 for Bigger Datasets. Mol Biol Evol **2016**; 33:1870–1874.

19. Almeida A, Villain A, Joubrel C, et al. Whole-Genome Comparison Uncovers Genomic Mutations between Group B Streptococci Sampled from Infected Newborns and Their Mothers. J Bacteriol **2015**; 197:3354–3366.

20. Treangen TJ, Ondov BD, Koren S, Phillippy AM. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol **2014**; 15:524.

21. O'Toole GA, Kolter R. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol **1998**; 28:449–461.

22. Siu LK, Fung C-P, Chang F-Y, et al. Molecular typing and virulence analysis of serotype K1 Klebsiella pneumoniae strains isolated from liver abscess patients and stool samples from noninfectious subjects in Hong Kong, Singapore, and Taiwan. J Clin Microbiol **2011**; 49:3761–3765.

23. Deleo FR, Chen L, Porcella SF, et al. Molecular dissection of the evolution of carbapenem-resistant multilocus sequence type 258 Klebsiella pneumoniae. Proc Natl Acad Sci U S A **2014**; 111:4988–4993.

24. Chen L, Mathema B, Pitout JDD, DeLeo FR, Kreiswirth BN. Epidemic Klebsiella pneumoniae ST258 Is a Hybrid Strain. mBio **2014**; 5:e01355-14-e01355-14.

25. Cannatelli A, Giani T, D'Andrea MM, et al. MgrB Inactivation Is a Common Mechanism of Colistin Resistance in KPC-Producing Klebsiella pneumoniae of Clinical Origin. Antimicrob Agents Chemother **2014**; 58:5696–5703.

26. Cannatelli A, D'Andrea MM, Giani T, et al. In Vivo Emergence of Colistin Resistance in Klebsiella pneumoniae Producing KPC-Type Carbapenemases Mediated by Insertional Inactivation of the PhoQ/PhoP mgrB Regulator. Antimicrob Agents Chemother **2013**; 57:5521–5526.

401 27. Olaitan AO, Morand S, Rolain J-M. Mechanisms of polymyxin resistance: acquired
402 and intrinsic resistance in bacteria. Antimicrob Resist Chemother **2014**; 5:643.
403 28. Casin I, Hanau-Bercot B, Podglajen I, Vahaboglu H, Collatz E. Salmonella enterica
404 Serovar Typhimurium blaPER-1-Carrying Plasmid pSTI1 Encodes an Extended-Spectrum
405 Aminoglycoside 6'-N-Acetyltransferase of Type Ib. Antimicrob Agents Chemother **2003**;
406 47:697–703.
407 29. Tzouvelekis LS, Miriagou V, Kotsakis SD, et al. KPC-Producing, Multidrug-Resistant
408 Klebsiella pneumoniae Sequence Type 258 as a Typical Opportunistic Pathogen. Antimicrob
409 Agents Chemother **2013**; 57:5144–5146.
410 30. Szijártó V, Guachalla LM, Hartl K, et al. Both clades of the epidemic KPC-producing
411 Klebsiella pneumoniae clone ST258 share a modified galactan O-antigen type. Int J Med
412 Microbiol **2016**; 306:89–98.
413 31. Naparstek L, Carmeli Y, Chmelnitsky I, Banin E, Navon-Venezia S. Reduced
414 susceptibility to chlorhexidine among extremely-drug-resistant strains of Klebsiella
415 pneumoniae. J Hosp Infect **2012**; 81:15–19.
416 32. Schroll C, Barken KB, Krogfelt KA, Struve C. Role of type 1 and type 3 fimbriae in
417 Klebsiella pneumoniae biofilm formation. BMC Microbiol **2010**; 10:179.
418 33. Alcántar-Curiel MD, Blackburn D, Saldaña Z, et al. Multi-functional analysis of
419 Klebsiella pneumoniae fimbrial types in adherence and biofilm formation. Virulence **2013**;
420 4:129–138.
421 34. Moradigaravand D, Martin V, Peacock SJ, Parkhill J. Evolution and Epidemiology of
422 Multidrug-Resistant Klebsiella pneumoniae in the United Kingdom and Ireland. mBio **2017**;
423 8.
424 35. Mathers AJ, Stoesser N, Sheppard AE, et al. Klebsiella pneumoniae Carbapenemase
425 (KPC)-Producing K. pneumoniae at a Single Institution: Insights into Endemicity from

426 Whole-Genome Sequencing. Antimicrob Agents Chemother **2015**; 59:1656–1663.
427 36. Arcilla MS, van Hattem JM, Matamoros S, et al. Dissemination of the mcr-1 colistin
428 resistance gene. Lancet Infect Dis **2016**; 16:147–149.
429 37. Liu Y-Y, Wang Y, Walsh TR, et al. Emergence of plasmid-mediated colistin
430 resistance mechanism MCR-1 in animals and human beings in China: a microbiological and
431 molecular biological study. Lancet Infect Dis **2016**; 16:161–168.
432 38. López-Rojas R, Domínguez-Herrera J, McConnell MJ, et al. Impaired Virulence and
433 In Vivo Fitness of Colistin-Resistant Acinetobacter baumannii. J Infect Dis **2011**; 203:545–
434 548.
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Figures Legends

Figure 1. Clinical and microbiological history of a patient colonized with a unique strain of KPC-Kp ST258 for 4,5 years A. 17 isolates were collected at different moments of patient's medical life. Kp BIC-1 designates the isolate collected from the endoscope. Known antimicrobial treatments are indicated in light grey boxes**. B.** Phylogenetic analysis of the 18 isolates. Numbers indicate the number of SNPs in the corresponding branch **C.** Molecular clock rate based on the linear association between the root-to-tip number of mutations and year of strain isolation. SNPs: Single Nucleotide Polymorphisms.

Figure 2. Serum bactericidal assay.

The average of survival (in %) of each strain in human serum is plotted against the incubation time (n=4, means ± SEM).° Kp53153 is a non-KPC non-ST258 strain. All strains were compared to the strain S17 (Student *t* test : *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 3. Biofilm formation.

A. Biofilm formation quantification using crystal violet staining. Absorbance values (n=4, means ± SEM) are indicated. The dotted line corresponds to the mean absorbance of KPC-Kp strains S1, S2, S3, S4, S5, S6, S8, S9, S10, S11, S12, S13, S14, S15, S16 and S17. Only the absorbance of the S7 strain (purple) is significantly increased compared to the other KPC-Kp strains (Student *t* test : ****, $p < 0.0001$). LM21 was used as a control positive strain [37]. In C-, no bacteria were added in the well. **B**. Schematic representation of the *fim* locus in the 18 isolates. The white and grey arrows indicate Right and Left Inverted Repeats respectively. **C.** Analysis of *fimH* expression using qRT-PCR (n=3, means of fold change expression ± SEM) (Student *t* test : ****, $p < 0.0001$).

462

463 **Table 1. Antimicrobial susceptibility testing of the 18 isolates.**

Name	Date	Origin	Susceptibility testing (mg/L)*					
			ERT[a]	IMI[a]	MER[a]	COL[b]	TGC[a]	GM[a]
Kp BIC-1	2009-09-25	endoscope	6	1.5	**4**	32	**1.5**	1.5
S1	2009-09-29	stool	12	1.5	**4**	128	**2**	1.5
S2	2009-10-12	stool	12	1.5	**4**	64	**1.5**	1.5
S3	2009-10-13	unknown	16	1.5	**6**	64	**2**	2
S4	2009-10-14	unknown	12	1.5	**4**	64	**1.5**	1.5
S5	2009-10-16	bile	12	1.5	**4**	32	**1.5**	1.5
S6	2009-11-03	stool	12	1.5	**4**	32	**2**	2
S7	2009-11-03	stool	8	1	**4**	32	**2**	12
S8	2009-11-11	urine	12	1	**4**	32	**2**	12
S9	2009-11-16	stool	12	1	**4**	64	**2**	12
S10	2009-11-21	urine	12	1	**4**	64	**1.5**	12
S11	2009-11-23	unknown	12	1	**4**	64	**1.5**	8
S12	2010-01-15	stool	16	1	**4**	64	**1.5**	2
S13	2011-07-29	stool	16	1	**4**	0.5	**2**	1.5
S14	2011-07-29	bile	24	2	**8**	0.5	**2**	1.5
S15	2012-08-23	blood culture	8	0.75	**3**	0.25	**1.5**	12
S16	2013-06-04	stool	24	1.5	**6**	0.5	**2**	1.5
S17	2014-04-29	BAL	12	1.5	**6**	0.5	**2**	1.5

464

465 ERT Ertapenem; IMI Imipenem; MEM Meropenem; COL Colistin; TGC Tigecyclin; GM
466 Gentamicin; BAL Bronchoalveolar lavage; [a] Performed with E-test; [b] Performed with broth
467 microdilution. * Resistant MIC values are highlighted in grey, intermediate MIC values are
468 written in bold.

469

470

Table 2. Effect of the SNP in *phoP* and *arnC* on colistin susceptibility

Strain	Susceptibility testing[1] Colistin
Kp BIC-1	32
S15*	0,25
S15*-pUC19	0,25
S15*-pUC19-*phoP*(WT)	64
S17#	0,5
S17#-pUC19	0,5
S17#-pUC19-*arnC*(WT)	32

[1]Determined by broth microdilution. Resistant MIC values are highlighted in grey.
* with the mutation PhoP I201N
# with the mutation ArnC C161Y

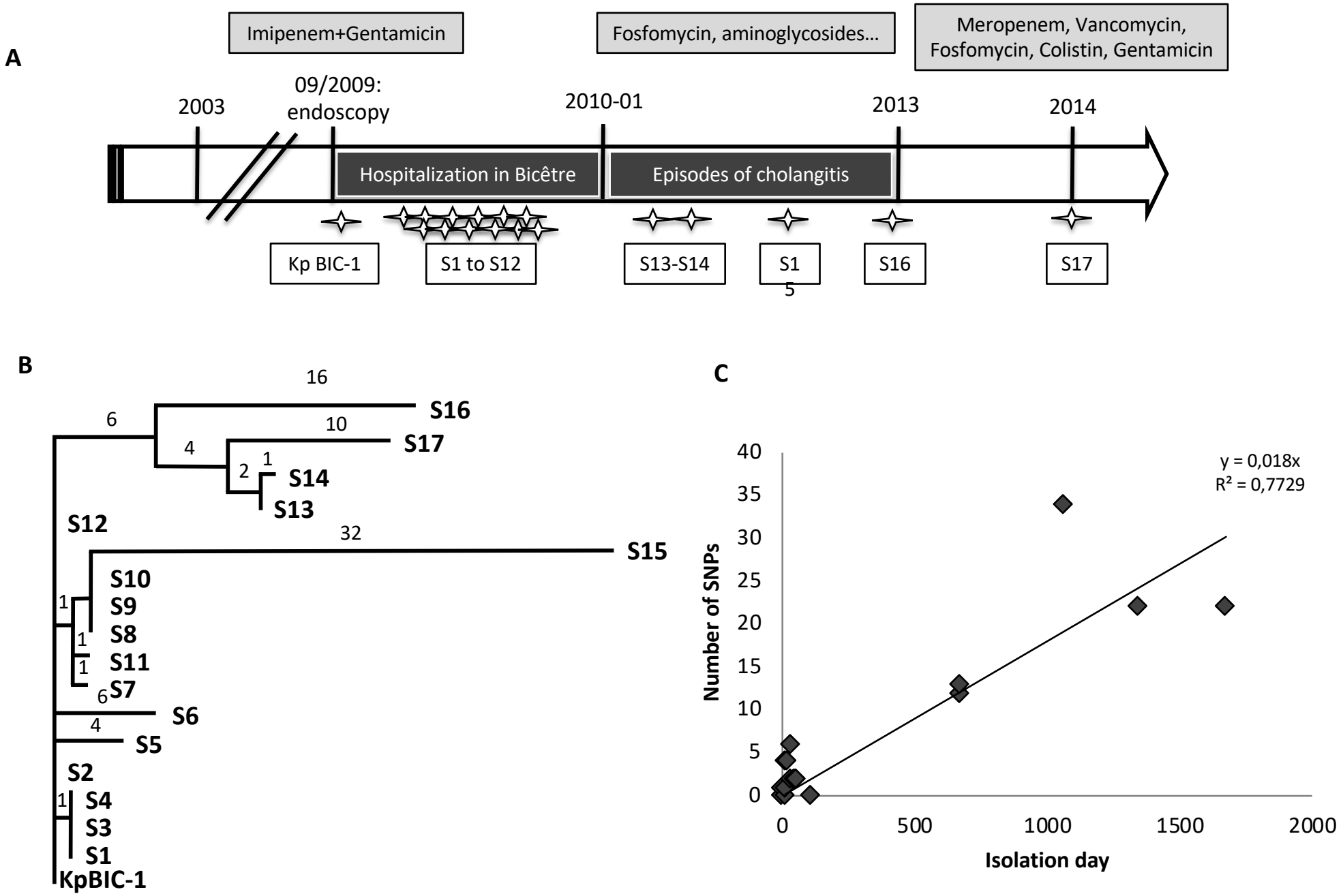
A
Imipenem+Gentamicin
Fosfomycin, aminoglycosides…
Meropenem, Vancomycin, Fosfomycin, Colistin, Gentamicin
2003
09/2009: endoscopy
2010-01
2013
2014
Hospitalization in Bicêtre
Episodes of cholangitis
Kp BIC-1
S1 to S12
S13-S14
S1
S16
S17
5
B
16
6
10
4
2
1
S16
S17
S14
S13
S12
32
S15
S10
S9
S8
S11
S7
6
S6
4
S5
S2
S4
S3
S1
KpBIC-1
C
y = 0,018x
R² = 0,7729
Number of SNPs
Isolation day
0
5
10
15
20
25
30
35
40
0
500
1000
1500
2000

Fig.1

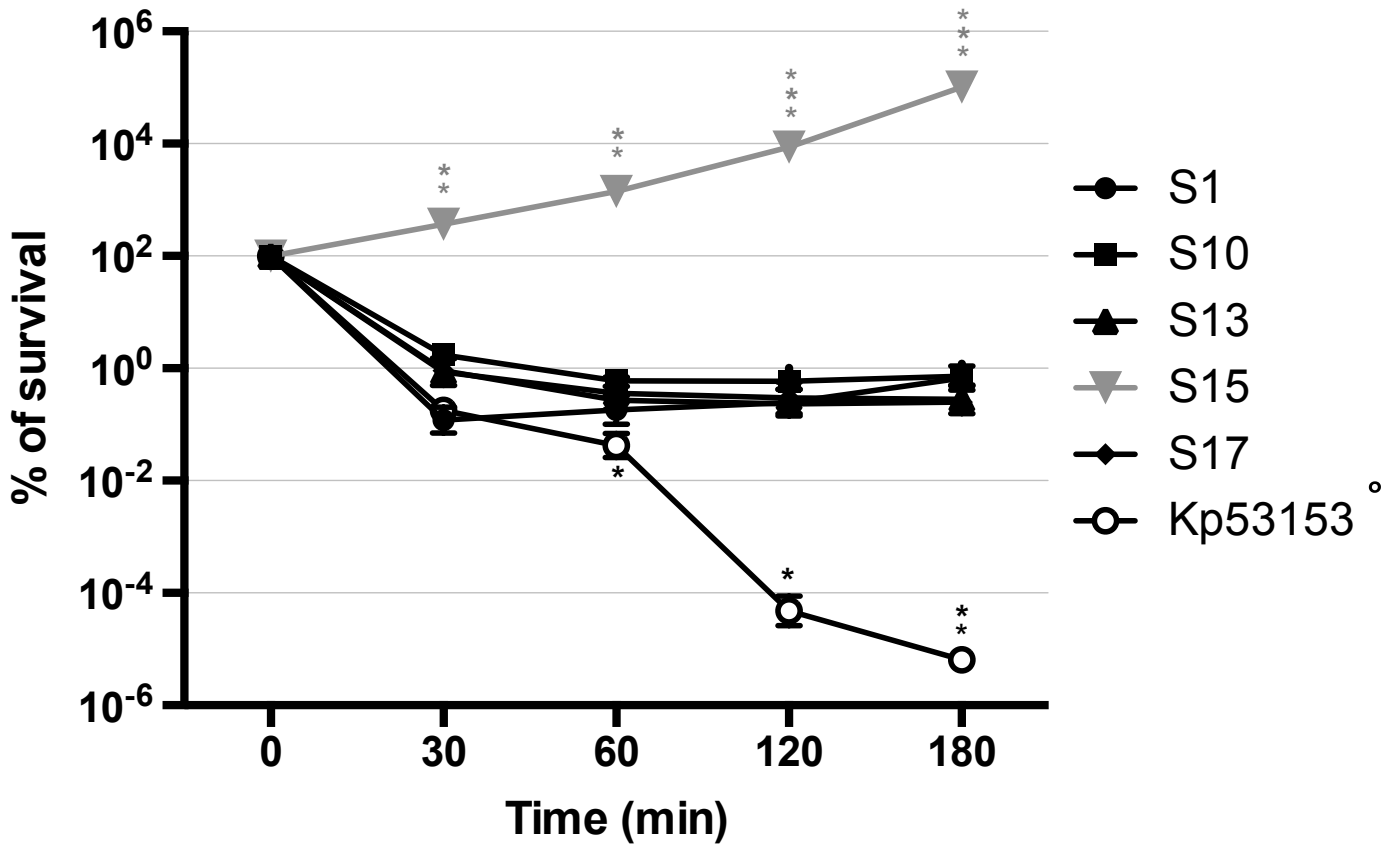
% of survival
10^6
10^4
10^2
10^0
10^{-2}
10^{-4}
10^{-6}
0
30
60
120
180
Time (min)
S1
S10
S13
S15
S17
Kp53153 °

Fig.2

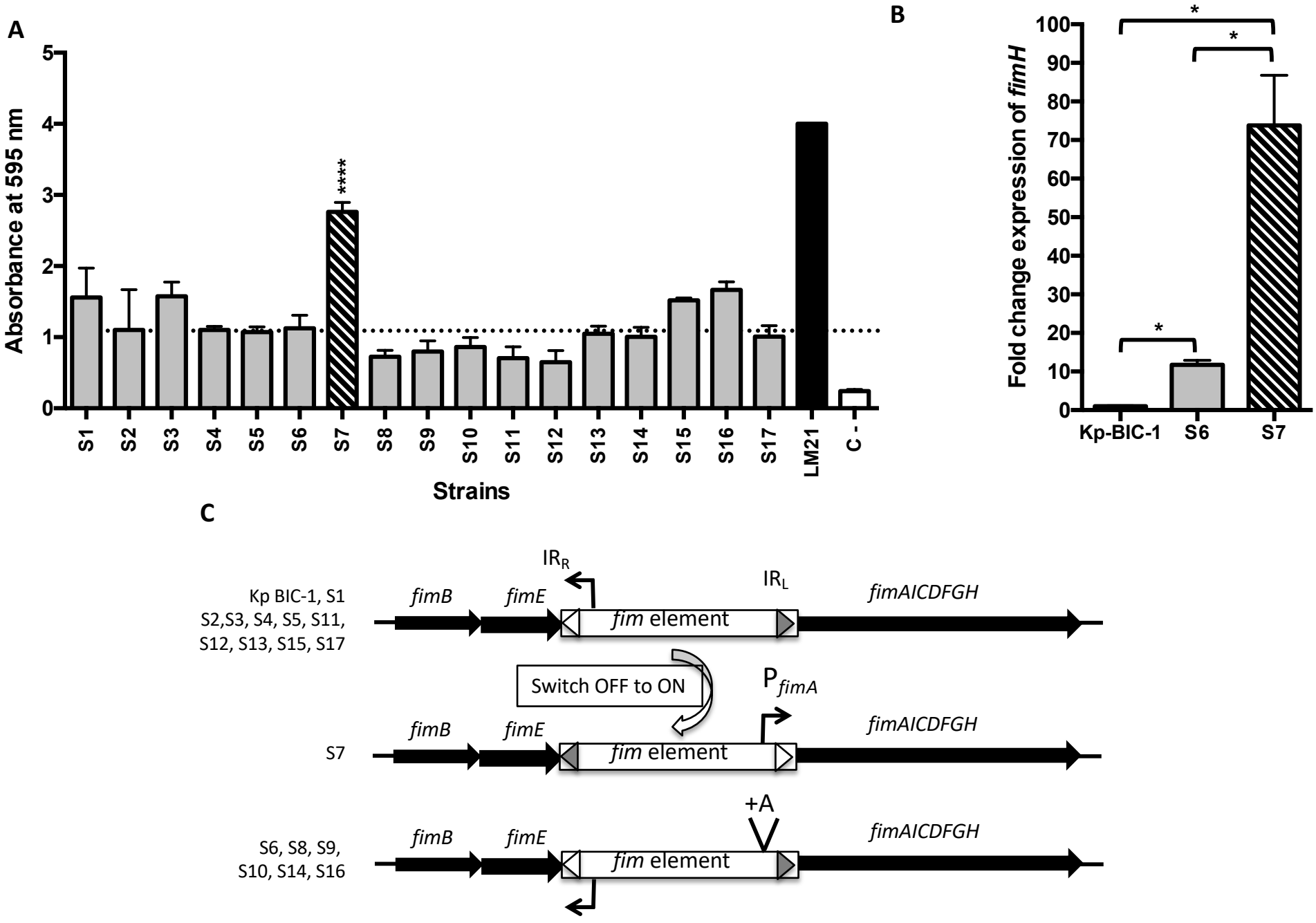
A
Absorbance at 595 nm
5
4
3
2
1
0
****
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
S12
S13
S14
S15
S16
S17
LM21
C -
Strains
B
Fold change expression of fimH
100
90
80
70
60
50
40
30
20
10
0
*
*
*
Kp-BIC-1
S6
S7
C
IR_R
IR_L
Kp BIC-1, S1
S2,S3, S4, S5, S11,
S12, S13, S15, S17
fimB
fimE
fim element
fimAICDFGH
Switch OFF to ON
P fimA
S7
fimB
fimE
fim element
fimAICDFGH
+A
S6, S8, S9,
S10, S14, S16
fimB
fimE
fim element
fimAICDFGH

Fig.3