Kpi, a chaperone-usher pili system associated with the worldwide-disseminated high-risk clone *Klebsiella pneumoniae* ST-15

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Control of infections caused by carbapenem-resistant *Klebsiella pneumoniae* continues to be challenging. The success of this pathogen is favored by its ability to acquire antimicrobial resistance and to spread and persist in both the environment and in humans. The emergence of clinically important clones, such as sequence types 11, 15, 101, and 258, has been reported worldwide. However, the mechanisms promoting the dissemination of such high-risk clones are unknown. Unraveling the factors that play a role in the pathobiology and epidemicity of *K. pneumoniae* is therefore important for managing infections. To address this issue, we studied a carbapenem-resistant ST-15 *K. pneumoniae* isolate (Kp3380) that displayed a remarkable adherent phenotype with abundant pilus-like structures. Genome sequencing enabled us to identify a chaperone-usher pili system (Kpi) in Kp3380. Analysis of a large *K. pneumoniae* population from 32 European countries showed that the Kpi system is associated with the ST-15 clone. Phylogenetic analysis of the operon revealed that Kpi belongs to the little-characterized \(\gamma_2\)-fimbrial clade. We demonstrate that Kpi contributes positively to the ability of *K. pneumoniae* to form biofilms and adhere to different host tissues. Moreover, the in vivo intestinal colonizing capacity of the Kpi-defective mutant was significantly reduced, as was its ability to infect *Galleria mellonella*. The findings provide information about the pathobiology and epidemicity of Kpi in *K. pneumoniae* and indicate that the presence of Kpi may explain the success of the ST-15 clone. Disrupting bacterial adherence to the intestinal surface could potentially target gastrointestinal colonization.

*Klebsiella pneumoniae* | GI tract colonization | ST-15 high-risk clone | pathogenesis | chaperone-usher pili system

**Significance**

Emergence of the pathogen *Klebsiella pneumoniae* (particu-
larly of carbapenem-resistant strains) is considered a dire threat to public health. Resistance and virulence determinants may favor the emergence of untreatable infections. Understanding the mechanisms involved in the pathogenesis and epidemicity of *K. pneumoniae* is essential for managing outbreaks and developing treatments. Here we identify a CUP system (Kpi) and infer the epidemiology of Kpi in *K. pneumoniae* in Europe. We demonstrate a direct link between Kpi presence and *K. pneumoniae* persistence in the hospital environment. Adherence of the bacterium to different cell types enables host colonization, favoring nosocomial outbreaks and spread of infection. Kpi appears to play a key role in the host-pathogen interaction and is associated with the worldwide-disseminated ST-15 clone.
factors that mediate the infectivity of the species. In addition to the advances in the application of carbapenemase inhibitors to treat these infections (9), monoclonal antibodies that act on _K. pneumoniae_ are also being developed and are showing promise as new therapeutic approaches or strategies (10, 11). In this respect, preclinical studies have demonstrated that the antibody KP3, which binds the type 3 fimbrial subunit, MrkA, may contribute to providing protection against MDR _K. pneumoniae_ (10–12). Targeting adherence-related virulence factors is a promising strategy as these factors may determine the capacity of a colonizing strain to cause infection. _K. pneumoniae_ expresses several fimbrial surface structures in order to adhere to abiotic and biotic surfaces. Chaperone-usher pili (CUP) systems are the most abundant adhesive structures in _K. pneumoniae_ and consist of one usher, at least one chaperone, and at least one fimbrial subunit. As CUP operons may have different numbers of chaperones and fimbrial subunits (13), they are classified on the basis of the usher protein into α-, β-, γ-, κ-, π-, and σ-fimbrial clades. Moreover, the γ-fimbrial elade is divided into γ1-, γ2-, γ3-, and γ4-fimbriae on the basis of specific operon features (14).

Up to nine different CUP systems have been identified in a single strain, apparently conferring the capacity to attach to different surfaces (15, 16). Almost all _K. pneumoniae_ isolates possess both classic types of CUP systems: Type 1 (fim) and type 3 (mrk) pili. Type 1 pili play a role in bacterial adherence to human mucosal or epithelial surfaces; although type 3 pili also attach to cell surfaces, they are considered important enhancers of biofilm formation (17). Type 1 pili are key pathogenic factors in _K. pneumoniae_ urinary tract infections (UTIs); however, their involvement in intestinal colonization or lung infection is unclear. It has recently been reported that deletion of the fim operon in an uropathogenic _Escherichia coli_ strain leads to significant defects in colonization (18). However, intestinal colonization and lung infection caused by _K. pneumoniae_ are not affected by either type 1 or type 3 pili (19, 20). In this study, we identified a CUP system in an epidemic carbapenem-resistant _K. pneumoniae_ strain. We demonstrate that this pilus system is involved in biofilm formation and adherence, as well as in the intestinal colonizing capacity of the pathogen. The system may therefore be considered a key pathogenic factor promoting the dissemination of _K. pneumoniae_. Analysis of a large and epide-miologically diverse _K. pneumoniae_ population revealed that all ST-15 isolates share the same Kpi operon identified in the outbreak-related Kp3380 strain. Thus, the presence of Kpi may explain the success of the ST-15 clone.

**Results**

**Clinical Impact of a Carbapenem-Resistant _K. pneumoniae_ Strain.** At A Coruña University Hospital from 2013 to 2018, patients with positive CRE clinical samples were routinely screened for intestinal colonization of CRE for infection control purposes and according to local guidelines. During the 6-γ-long study, 5,340 _K. pneumoniae_ nonduplicate isolates were detected in clinical and colonization samples in the Microbiology Department. Approximately a 13% (682 of 5,340) of these isolates were positive for OXA-48 carbapenemase, thereby dramatically increasing the prevalence of carbapenem-resistant isolates. Genotyping showed that most of the isolates belonged to an ST-15 clone, which was initially isolated from 15 patients in 2015 and spread rapidly, causing a large outbreak despite the implementation of strict control measures. We tested intestinal colonization in 462 of 682 patients infected or colonized with the OXA-48-producing _K. pneumoniae_ strain. A 93.3% of them were positive for this epidemic clone. To understand the success of the ST-15 clone, we conducted an in-depth characterization of the associated virulence functions.

**The Epidemic Clone Kp3380 Is Highly Adherent.** The Kp3380 isolate was used as a representative clone of the above-mentioned outbreak. We used eukaryotic epithelial cells (HT-29 colorectal cells, H1-1376 bladder cells, and A549 alveolar cells) to analyze the adherence phenotype of the clinical strains Kp3380, Kp90107, Kp924, Kp727, Kp1278, along with the reference strain KMH78578 and ATCC10031, which represent different host cells targeted by _K. pneumoniae_. The data collected (Fig. 1A) revealed that Kp3380 and MGH78578 had a higher capacity for adherence than the other strains tested (P < 0.0001). Similarly, strain Kp3380 demonstrated a hyperbiofilm-producing phenotype, reaching an OD of ~4.5 (as assessed by Crystal violet staining), which is significantly different (P < 0.0001) from that of most of the strains assayed (i.e., OD of 0.6 to 1.3) (Fig. 2A). We wondered whether the differences in adherence shown by these strains could be explained by bacterial surface structures used in cell adhesion processes. To address this question, we cultured strains under the same conditions used to infect the human epithelial cells and examined the cells by transmission electron microscopy (TEM). Microscopic observation of the surface appendages produced by these strains revealed quantitative and qualitative differences between the strains analyzed (Fig. 1B). We observed a relationship between the presence of abundant pilus-like structures on the bacterial surface and a highly adherent phenotype.

**Characterization of a CUP System in Kp3380.** Protein coding genes involved in adherence were analyzed relative to the fully sequenced, annotated genome of clinical isolate Kp3380 (21). Comparative genome analysis enabled us to identify a region of ~6,500 bp containing seven genes, which are thought to be involved in adherence. In silico analysis of the genomic region revealed that it may code for a polycistrionic operon. RT-PCR analysis of total RNA demonstrated that genes CWR42_04160 to CWR42_04190 are cotranscribed as a single operon. Intergenic fragments amplified from cDNA match those observed when genomic DNA was used as a template (SI Appendix, Fig. S1). The operon, named here kpiABCDEFG, consists of one usher gene (kpiG), three molecular chaperone genes (kpiB, kpiE, and kpiF), and three fimbrial genes (kpiA, kpiC, and kpiD) (Fig. 3A). The function of each gene was predicted on the basis of amino acid homologies with other fimbrial proteins. The protein structure of each gene product was predicted using the RaptorX server. Each protein belonging to the Kpi pilus system was used as a query to search for related protein structures included in the Protein Database. The results are summarized in SI Appendix, Table S1. Protein structure modeling revealed that the Kpi pilus system is structurally related to CUP system type 1 (SI Appendix, Table S1). However, the phylogenetic analysis based on the usher (KpiG) amino acid sequence comparisons (SI Appendix, Table S2) showed that Kpi is included in the phylogenetic clad γ3 (Fig. 3B).

A more detailed functional analysis of each protein of the Kpi system was conducted using the InterPro and HHpred servers. Three conserved usher domains were identified in KpiG: PF00577 (amino acids 203 to 767), PFAM13954 (amino acids 37 to 182), and PFAM13953 (amino acids 776 to 842) (Fig. 3A). HHpred analysis revealed that all fimbrial proteins contain the conserved domains COG3539 and PFAM00414. However, the domain PFAM00419 was only found in KpiC and KpiD (amino acids 54 to 205 and 38 to 191, respectively) when using InterPro server (Fig. 3A). All three chaperones (KpiB, KpiE, and KpiF) were found to contain two conserved chaperone domains: PFAM00345 (amino acids 24 to 154, 4 to 172, and 3 to 163, respectively) and PFAM02753 (amino acids 176 to 205 and 38 to 191, respectively) (Fig. 3A). Comparative functional and sequencing analysis of the proteins of the Kpi and the γ3-fimbriae Fas (14).
revealed that KpiD might be the major fimbrial subunit as it shares the highest sequence identity (21.76% identity and 96% coverage) with FasA. All three chaperones of the Kpi system (KpiB, KpiE, and KpiF) only share sequence identity with FasB (22.41% identity and 98% coverage, 24.12% identity and 85% coverage, 21.63% identity, and 91% coverage, respectively).

Fig. 1. Adherence phenotype and TEM images of K. pneumoniae. (A and C) Quantification of the adherence of K. pneumoniae strains to HT-29 intestinal colorectal cells, HT-1376 bladder cells, and A549 alveolar cells. Bacterial adherence is reported as the percentage of attached bacteria relative to the number of bacteria used to infect the eukaryotic cells (assumed to be 100%). The data represent four independent biological replicates. ANOVA was applied to the data to indicate significant differences between the groups, and a post hoc Tukey’s test was used to determine the difference between each group and the respective control (Kp3380 in A and Kp3380ΔkpiD in C). Statistical significance is indicated (*P ≤ 0.05, **P ≤ 0.01, and ****P ≤ 0.0001). Values shown are means, and error bars indicate SDs. (B and D) TEM images of K. pneumoniae strains. Micrographs were taken at 30,000× (B), 60,000× (D, Upper), and 250,000× (D, Lower) magnification. Bars indicate the scale.
The Kpi Fimbriae System Is Closely Associated with the High-Risk Clone *K. pneumoniae* ST-15. We analyzed the genomes of 1,649 *K. pneumoniae* isolates collected from 32 European countries as part of the European Survey of Carbapenemase-Producing Enterobacteriaceae (EuSCAPE), in order to evaluate the distribution of the Kpi system in *K. pneumoniae*. The Kpi system was detected in 19.1% (315 of 1,649) of isolates belonging to 41 different STs. In addition, 46% (145 of 315 isolates) of Kpi+ *K. pneumoniae* belonged to ST-15 (Fig. 4), and all of the ST-15 isolates tested positive for the Kpi system. A total of 132 (91.1%) ST-15 isolates shared exactly the same operon with Kp3380 (100% coverage and 100% identity) (Fig. 4). Seven (4.8%) ST-15 isolates had several amino acidic changes, and in six (4.1%) of the ST-15 isolates the whole operon could not be evaluated because of a gap in the genome sequence (between two different contigs) (Fig. 4). The Kpi system was also detected in 16.8% (53 of 315 isolates), 9.5% (30 of 315 isolates), and 4.7% (15 of 315 isolates) of *K. pneumoniae* isolates belonging to ST-405, ST-14, and ST-25, respectively (Fig. 4). However, the *kpiABCDEFG* operon from ST-14, ST-25, and ST-405 isolates displayed several amino acid changes relative to the Kp3380 strain (Fig. 4).

Examination of the distribution of ST-15 isolates showed that this clone was involved in outbreaks occurring in Hungary, Croatia, Romania, and Spain (Fig. 4). In addition, the presence of Kpi was evaluated in all of the genomes of *K. pneumoniae* deposited in GenBank (7,950 genomes), in order to confirm that all ST-15 isolates are positive for the system. Of these genomes, 4.7% (377 of 7,950) of the isolates belonged to ST-15. In addition, 98.1% (370 of 377) of the isolates were Kpi+ and shared the same operon with Kp3380 (100% coverage and 98 to 100% identity). In 1.89% (7 of 377) of the ST-15 isolates, the whole operon could not be evaluated, because of a gap in the genome sequence (between two different contigs). Thus, although the percentage of ST-15 *K. pneumoniae* isolates in all of the genomes available in GenBank was lower, the results are consistent with those obtained with the EuSCAPE collection.

The Presence of the Kpi Fimbriae System Is Associated with Adherence. DNA-based multiple comparison of the operon *kpiABCDEFG* in isolates Kp09107 (ST-101), Kp727 (ST-405), Kp924 (ST-11), Kp1278 (ST-15), Kp3380 (ST-15), and MGH78578...
revealed that only Kp3380 and MGH78578 have a complete, apparently functional operon. Analysis of the nucleotide identity of Kpi in MGH78578 revealed several amino acid changes in the operon relative to the Kp3380 strain. Although strain Kp727 has a complete operon, it has multiple stop codons in the kpiD gene. Strain Kp1278 only had the kpiG gene, which codes for the usher protein. Neither of these genes was found in strains Kp924 or Kp09107.

We also analyzed the most common adhesive appendages in K. pneumoniae: That is, the type 1 and type 3 fimbriae (pili), in all of the above-mentioned strains. We found that all strains have intact type 1 (fim) and type 3 (mrk) loci.

We observed a correlation between the presence of the Kpi system and a highly adherent phenotype. Interestingly, we observed a nonadherent phenotype in the Kp727 strain, which harbors a complete operon with multiple stop codons in the kpiD gene. Therefore, deletion of the fimbrial protein KpiD seems to be crucial for the functional inactivation of the Kpi system.

Effect of kpiD Gene Deletion on Attachment to Eukaryotic Cells.

Considering that the fimbrial protein KpiD plays an important role in the production of functional fimbriae in the Kp727 K. pneumoniae strain and it might be the major subunit of the Kpi system, we deleted the kpiD gene to investigate the role of Kpi fimbriae in bacterial functions. A kpiD-defective knockout strain (Kp3380ΔkpiD) was constructed and the parental kpiD gene was introduced into Kp3380 ΔkpiD for complementation.

The interactions between Kp3380 (and derivative strains) and eukaryotic cells were also examined. For this purpose, different host cells frequently targeted by K. pneumoniae—including colorectal...
cells (HT-29), bladder cells (HT-1376), and alveolar cells (A549)—were infected with Kp3380, Kp3380ΔkpiD, and the complemented strain (Kp3380ΔkpiD_C) to determine their adherent phenotype and thus demonstrate the role of the kpiD gene in adherence. This approach revealed that significantly fewer Kp3380ΔkpiD bacteria were recovered from infected cells than from cells infected with Kp3380 bacteria, regardless of the type of host cells tested. Absence of the kpiD gene reduced the number of bacteria attached to HT-29, HT-1376, and A549 eukaryotic cells by, respectively, ∼50% (P ≤ 0.01), 60% (P ≤ 0.05), and 40% (P ≤ 0.01) (Fig. 1C). The

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**Fig. 4.** International spread of Kpi+ K. pneumoniae isolates. The phylogenetic tree includes 1,649 K. pneumoniae isolates collected during the EuSCAPE survey. The colors of the isolates indicate absence/presence of the kpiABCDEFG operon and whether or not the operon has amino acidic changes or the sequence is incomplete. Blue shadow indicates that isolates are genetically related.
parental phenotype was restored in the complemented strain (Kp3380ΔkpiD_C) (Fig. 1C).

Considering that Kp3380 loses its highly adherent phenotype when the kpiD gene is deleted, we wondered whether the production of bacterial surface structures in this mutant strain would be affected. TEM images were obtained from the bacterial surface of the wild-type strain and its derivative strains, cultured under the same culture conditions used to infect the eukaryotic cells. Strain Kp3380 and the Kp3380ΔkpiD-complemented strain produced abundant pilus-like structures around the cell surface (Fig. 1D). In contrast, TEM revealed that there were no pilus-like structures around the mutant cells (Fig. 1D).

Deletion of the kpiD Fimbriae Gene Reduces Biofilm Formation. Two different approaches were used to test the effect of kpiD deletion on biofilm formation. The Crystal violet assay revealed that inactivation of the kpiD gene significantly reduced biofilm production (P ≤ 0.0001) (Fig. 2B). Complementation of the knockout strain with the parental allele (Kp3380ΔkpiD_C) restored the wild-type phenotype (Fig. 2B). Biofilms produced by the strains of interest were also evaluated by confocal laser-scanning microscopy (CLSM) and digital imaging. Fig. 2C shows biomass measurements as well as representative 3D renderings of the biofilms. Quantification using reconstructions of confocal sections revealed minimal differences between live and dead bacteria in comparisons of biofilms formed by the wild-type strain and the knockout strain. However, the Kp3380ΔkpiD strain formed significantly less biofilm, which was also structurally altered (Fig. 2C). Complementation of the mutant strain (Kp3380ΔkpiD_C) restored the parental phenotype. The biofilm formed by the complemented strain showed a structure similar to that of Kp3380.

Kp3380ΔkpiD Is Attenuated In Vivo. A Galleria mellonella survival assay (16 larvae per group) was used to assess the role of the kpiD gene in the virulence of K. pneumoniae Kp3380. The survival study (Fig. 5A) showed that 84% of the larvae infected with the parental strain died after 5 d of infection, while 50% of the larvae infected with the kp3380ΔkpiD mutant survived. The ability of the mutant strain to infect and kill G. mellonella was therefore significantly affected (P = 0.0046), relative to that of the wild-type strain. Larvae injected with sterile PBS were used as negative controls.

The parental phenotype was not successfully restored in the complemented strain, as it did not retain the complementation plasmid in the in vivo model (59% of bacteria did not harbor the plasmid at 15 h postinfection).

Role of Kpi Fimbriae System in Kp3380 Intestinal Colonization and Lung Infection. Considering the role of the Kpi fimbriae system in in vitro adherence to HT-29 colorectal cell and A549 alveolar cells, we used in vivo mice models to investigate the role the system plays in both intestinal colonization and lung infection. The in vivo colonization ability of strain Kp3380 lacking the kpiD gene was investigated. Mice pretreated with streptomycin (six per group) (Fig. 5B) were individually inoculated (via oral route) with either the parental strain Kp3380 (3.8 × 10⁸ CFU/mL PBS) or the mutant strain Kp3380ΔkpiD (4.1 × 10⁶ CFU/mL PBS). The fecal bacterial burden 24 h after colonization was 2.33 × 10⁶ CFU/mL for Kp3380 and 5.5 × 10⁶ CFU/mL for Kp3380ΔkpiD. Colonized mice were killed on day 3 after inoculation to enable determination of the intestinal bacterial burden. The levels of bacteria were significantly lower (P = 0.0022) in the cecum and colon of mice colonized with the mutant strain than in the corresponding organs of mice colonized with the wild-type strain (Fig. 5B). Mice orally inoculated with PBS were used as negative controls, and no bacteria were recovered from intestinal segments of these mice. The parental

Fig. 5. Role of the kpiD gene in K. pneumoniae virulence. (A) G. mellonella larvae were injected with Kp3380 or Kp3380ΔkpiD bacteria and survival was monitored for 5 d. Larvae injected with PBS were used as negative controls. The log-rank (Mantel–Cox) test was used to compare the survival distributions of two groups: The larvae infected with Kp3380 and the larvae infected with Kp3380ΔkpiD. The statistical significance is indicated (**P = 0.0096). (B) Intestinal colonization of mice with the Kp3380 parental strain or the Kp3380ΔkpiD mutant was achieved by oral gavage with −10⁸ bacteria. The bacterial burden in intestinal segments (cecum and colon) was determined 72 h postinoculation. The Mann–Whitney test was used to determine significant differences between the two groups. The statistical significance is indicated (**P = 0.0022). Values are means, and bars indicate the SD. (C) Mice were intratracheally infected with −6 × 10⁸ of the Kp3380 parental strain or the Kp3380ΔkpiD mutant. The number of bacteria in lung homogenates was determined 24 h postinfection. The Mann–Whitney test was used to determine significant differences between the groups. Not statistically significant differences were observed (NS). Values are means, and bars indicate the SDs.
phenotype was not successfully restored in the complemented strain, as it did not retain the complementation plasmid in the in vivo model (45% of bacteria did not harbor the plasmid on day 3 after inoculation).

Similarly, mice were intratracheally infected with the Kp3380 and its kpiD-defective mutant to assess the in vivo virulence of this strain in a lung infection model. At 24 h postinfection the mice infected with Kp3380kpiD had a similar lung burden as the mice infected with the parental strain (Fig. 5C).

Discussion

The severity, prevalence, and morbidity of carbapenem-resistant K. pneumoniae infections manifest the urgent need for new strategies to manage and treat these serious infections. A large number of MDR and extensively drug-resistant K. pneumoniae strains belonging to specific clonal groups that have emerged and have become globally disseminated show an enhanced capacity to cause outbreaks worldwide. Clonal group 15, consisting of ST-14 and ST-15, is one of the most significant outbreak-causing clonal groups. Understanding the epidemic traits of these highly disseminated successful clones is essential to enable identification of specific targets for developing preventive measures and treatment (4). Antimicrobial resistance and virulence are the main factors favoring the successful emergence of these high-risk clones.

A number of virulence factors have been demonstrated to promote K. pneumoniae infectivity (17). In this study, we used an outbreak-related ST-15 K. pneumoniae isolate to identify potential pathogenic targets in K. pneumoniae. Phenotypic analysis revealed that this strain has a more adherent phenotype than other strains belonging to different STs from outbreaks in other hospitals in Spain. Electron microscopy data revealed a re-organization. Unlike the Fim system, Kpi belongs to the largely unexplored γ longing to family. γ-longing to family 2-fimbriae [i.e., Fas in E. coli (14)] revealed that these systems share common characteristics. The fimbrial protein KpiD is possibly the major subunit. KpiA shares the functional annotation characteristics with FasG; therefore, we hypothesized that this protein could be the tip adhesin. However, KpiA is shorter than FasG and is not structurally related to any crystallized protein. However, the Kpi system does not share the same gene organization. Unlike the Fim system, Kpi belongs to the largely unexplored γ 2-fimbrial clade. Like the Kpi system, operons belonging to γ 2-fimbriae have three chaperone genes (14) and share similar gene organization. Comparative functional and sequencing analysis of Kpi and the best-studied member of the γ 2-fimbriae [i.e., Fas in E. coli (14)] revealed that these systems share common characteristics. The fimbrial protein KpiD is possibly the major subunit. KpiA shares the functional annotation characteristics with FasG; therefore, we hypothesized that this protein could be the tip adhesin. However, KpiA is shorter than FasG and is not structurally related to any crystallized protein, which pointed out that additional analyses are required to elucidate the function of this protein. Unlike the Fas system, all three chaperones of the Kpi system contain the two chaperone domains: PFAM00345 and PFAM02753. Although Kpi is phylogenetically related to the Fas system, it is phylogenetically more closely related to the uncharacterized member pilu2068 from Photorhabdus luminescens (14).

Several CUP systems, including the type 1 and type 3 fimbriae (pili) (15–17), have been reported to be involved in adherence in K. pneumoniae, and we therefore wondered whether the ability of Kp3380 to form biofilms and to attach to eukaryotic cells was due to the presence of the Kpi system.

Although the Kpi pilus system is structurally related to the type 1 pili system, it plays a role in the interaction of Kp3380 with both abiotic and biotic surfaces. Like their Fim (7) and Kpg (15) counterparts, Kpi is needed for adherence to epithelial surfaces. However, unlike Fim fimbriae, the Kpi system has been demonstrated to be required for the attachment of Kp3380 to different types of epithelial cells, such as HT-29 intestinal cells, A549 alveolar cells, and HT-1376 bladder cells. Similarly, the ability of the Kp3380 strain to form biofilms has been shown to be impaired when the fimbrial protein KpiD was deleted. A number of CUP systems have been reported to play a role in biofilm formation, highlighting the role of type 3 (murk) pili as a strong biofilm promoter (22–26). Type 1 (fim) pili, along with type 3 (murk) pili, are involved in biofilm formation on urethral tract catheters (23–27). Up to eight usher-type pili have been identified in the LM21 K. pneumoniae strain, among which Kpa, Kpg, and Mrk also play a role in biofilm formation (15). Similarly, Kpc fimbriae have been shown to increase biofilm formation activity in the K. pneumoniae NTUH-K2044 strain (16).

In view of the fact that Kpi is involved in adherence to coloecal cells, and that the first step in most infections caused by K. pneumoniae is colonization of the patient’s GI tract, we used an in vivo animal model to investigate the role of the fimbriae in intestinal colonization. The Fas system is involved in adherence to piglet intestinal cells in E. coli (14). Other adhesives structures have been reported to confer binding to intestinal cells in K. pneumoniae, suggesting that they play a role in GI tract colonization (28–30). However, the role of type 1 in type 3 fimbriae in intestinal colonization remains controversial. It has been reported that neither type of pilus affects the ability of K. pneumoniae to colonize or infect the lungs (20, 27, 31). In contrast to previous reports, it has been claimed that murine intestinal colonization is impaired in both mrk-defective LM21 and fim-defective LM21 strains (15). It has recently been reported that down-regulation of type 1 fimbriae in K. pneumoniae NTUH-K2044, together with the lack of type VI secretion system, is important for bacterial intestinal colonization (32).

We have demonstrated here that the colonizing capacity of the KpiD-defective Kp3380 strain was significantly reduced, indicating that the fimbriae are required for GI tract colonization. However, we did not observe attenuation of the virulence of the Kp3380kpiD mutant strain in an acute murine pneumonia infection model. The observed differences in these two in vivo models may be due to the different host environment. Bacteria using different interacting mechanisms with the host may use distinct strategies when interacting with hosts, and the molecular mechanisms used by K. pneumoniae to colonize the GI tract or to cause acute infections, such as pneumonia, may differ widely. Adherence-related virulence factors (such as Kpi) in K. pneumoniae may play a crucial role in colonization while other virulence factors may have a more important role in acute infection models. As colonization with K. pneumoniae is closely associated with subsequent infection, our findings may be important for understanding the underlying mechanisms of disease progression. Kpi+ K. pneumoniae may have an advantage that enables them to persist in humans and in the hospital environment, thus favoring progression of the outbreak, despite intensive intervention protocols. Indeed, we found that this CUP system is closely associated with clonal group 15, which includes widely disseminated high-risk clones of K. pneumoniae causing outbreaks worldwide (4). Therefore, the Kpi system may at least partly explain the superior capacity of these clones to spread and cause outbreaks and may represent a specific target for controlling the emergence of these successful high-risk clones.

Finally, as the ability of the mutant strain to infect and kill G. mellonella was significantly affected, along with its role in adherence and biofilm formation, we suggest that Kpi plays a role in the virulence of K. pneumoniae Kp3380.

It is worth pointing out that, contrary to what we found in the in vitro assays, plasmid-based complementation of the Kpi system did not restore wild-type function in the in vivo models, despite roughly half of the bacteria harboring the complementation plasmid.
A possible explanation for this might be that the plasmid-carrying bacteria are outcompeted by plasmid-free bacteria, due to the fitness cost associated with plasmid expression (33, 34), particularly under the growth-limiting conditions found in the host environment. This fact, along with the observed loss of complementation plasmid, might be decisive in the plasmid-bearing bacteria being unable to develop the infection properly, leading to the complementation failure.

In summary, we identified a CUP system associated with the worldwide-disseminated high-risk clone \(K. pneumoniae\) ST-15. Our findings provide evidence that this system positively contributes to \(K. pneumoniae\) adherence, biofilm formation, and virulence in specific models. The information acquired regarding the pathobiology and epidemiology of Kpi\(^+\) \(K. pneumoniae\) indicates that the Kpi system could be targeted in order to interrupt host-pathogen-interactions that promote dissemination of the \(K. pneumoniae\) ST-15 clone.

**Materials and Methods**

Screening of Carbapenemase-Producing \(K. pneumoniae\). Infection prevention and control interventions in our hospital include screening of CRE colonized patients when a positive clinical sample for CRE is detected (35). First, in- testinal colonization (rectal swabs) is analyzed for patients infected with CRE (clinical sample positive for CRE). Then, screening of patients admitted to the same room, as well as those patients admitted to the same department, are also included in the screening, according to the infection control protocols of our hospital. Patients from whom CRE have been isolated from a clinical sample are placed in strict contact isolation until two consecutive rectal swabs are negative for CRE. These patients are then discharged from contact isolation measures. None of the authors has any contact with patients. The samples were handled under strict confidentiality criteria without any possibility of relating a bacterial isolate to any patient.

**Multilocus Sequence Typing Analysis.** Multilocus sequence typing (MLST) was performed using seven housekeeping genes (gapA, infB, mdh, pgi, phoE, rpoB, and tonB) as previously described (36). Sequences of amplified genes were used to obtain the MLST profile using the \(K. pneumoniae\) MLST database (https://bigd.bigpasteru.fr/klebsiella/klebsiella.html).

**Bacterial Strains, Plasmids, and Culture Conditions.** The bacterial strains and plasmids used in this study are listed in \textit{SI Appendix, Table S3}. Strain Kp3380 was selected from a collection of 682 \(K. pneumoniae\) outbreak-related isolates obtained between February 2013 and October 2018. Isolates were selected on the basis of reduced susceptibility to ertapenem. All isolates were obtained between February 2013 and October 2018. Isolates were sequenced on the basis of reduced susceptibility to ertapenem. All isolates were obtained between February 2013 and October 2018.

**RNA Extraction and RT-PCR.** To demonstrate the polyclonal nature of the operon, the cDNA was obtained from RNA samples with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s recommendations. RNA was obtained using the High Pure RNA Isolation Kit (Roche). RNA samples were treated with DNase I and purified with RNeasy MiniElute Cleanup Kit (Qiagen). The cDNA was amplified with GoTaq.

For DNA polymerase (Promega), pairs of primers were designed to be complementary to the 3' end of every gene and the 5' end of the next one. Genomic DNA was used as a template for the positive control and total RNA was used as a template for the negative control of the amplification. The primers and probes used are listed in \textit{SI Appendix, Table S4}.

**Bioinformatics Analysis.** Rapid Annotation with Subsystem Technology (RAST) (https://rast.nmpdr.org) was used for comparative analysis of \(K. pneumoniae\) genomes. Functional annotation of proteins of the CUP system \(KpiABCDEFG\) was assessed using InterPro and HHpred (37, 38). Protein structure modeling of the CUP system \(KpiABCDEFG\) in \(K. pneumoniae\) was predicted using RaptorX (RaptorX: Protein Structure and Function Prediction Powered by Deep Learning, raptorx.uchicago.edu).

**Phylogenetic Analyses.** Phylogenetic analysis based on the usher amino acid sequence was used to classify the \(kpiABCDEFG\) fimbrial operon. A set of reference proteins (\textit{SI Appendix, Table S2}) was used to establish the \(T_v\) and \(T_g\) groups (14).

A hidden Markov model (HMM) was built using the seed alignment of the Pf00577 family from Pfam (user family), with hmmbuild in the HMMER v3.0 package (39). The usher set (KpiG) and the proteins listed in \textit{SI Appendix, Table S2} were aligned against the HMM by using hmmpalin in the HMMER v3.0 package, with the outtophyl pioph (39). The resulting alignment was used in the phylogenetic analysis. Maximum-likelihood (ML) phylogeny was constructed using RaXML v8.0 (40) and the LG model of amino acid substitution rates with empirical amino acid frequencies and the \(\gamma\)-model of rate heterogeneity (–m PROTGGAMMALGF option). A rapid Bootstrap analysis and search for the best scoring ML tree was performed (–f option) with 1,000 repetitions (the random number seed for the parsimony inferences was –p 12345 and for rapid bootstrapping, –x 12345). The best tree graphic representation was generated using iTOL v5.1.1 (41).

**Construction of the Knockout Strain.** The \(kpiD\) gene was deleted from Kp3380 by a previously described method (43). Briefly, the pIU773 plasmid was used as a template to amplify the apramycin resistance cassette flanked by FRT sites with primers that included 5' extensions with homology for the \(kpiD\) gene. (\textit{SI Appendix, Table S3}). The \(kpiD\) gene was PCR-amplified with specific oligonucleotides (\textit{SI Appendix, Table S4}). The product was electroporated into Kp3380 previously transformed with the plasmid pACBSS2-Hyg, which expresses the \(\lambda\)-Red system and a hygromycin selection marker. Bacteria were incubated overnight on LB Agar supplemented with apramycin, and correct insertion of the apramycin cassette was confirmed by PCR. The Kp3380 derivative strain was grown on LB agar with apramycin (150 \(\mu\)g/mL) at 37 °C for 3 d, and then incubated on LB agar with apramycin (150 \(\mu\)g/mL) and LB agar with hygromycin (200 \(\mu\)g/mL) overnight at 37 °C to test for loss of the helper plasmid. For removal of resistance markers, cells were transformed with pLP-Hyg and grown overnight at 30 °C. The mutants were then incubated at 43 °C to remove the apramycin resistance cassette. Apramycin-susceptible mutants were selected to confirm the \(kpiD\) gene deletion by PCR and sequencing. The level of expression of each gene in the \(kpiABCDEFG\) operon in each generation was confirmed by PCR to confirm that only the \(kpiD\) gene expression was null in the Kp3380ΔkpiD strain. The primers used in this procedure are listed in \textit{SI Appendix, Table S4}.

**Complementation of the Mutant Derivative Kp3380ΔkpiD Strain.** The fimbrial gene \(kpiD\) was PCR-amplified with specific oligonucleotides (\textit{SI Appendix, Table S4}) from the Kp3380 strain. The amplified DNA was purified, digested with SmaI and SacI (Thermo Fisher Scientific), and then ligated (T4 DNA ligase, Thermo Fisher Scientific) into a similarly digested expression pUCP24 vector under control of the tetracycline gene promoter. The tetracycline gene promoter was first amplified from the plwH1266 plasmid, digested with BamHI and SmaI endonucleases (Thermo Fisher Scientific) and then cloned into the pUCP24 vector. The construct was checked by PCR analysis with specific primers (\textit{SI Appendix, Table S4}). The derivative pUCP24/\(kpiD\) plasmid was incorporated into the Kp3380ΔkpiD mutant strain by electroporation for complementation studies. Transformant strains were selected on agar plates with gentamicin (50 \(\mu\)g/mL).

**Bacterial Adhesion to Human Epithelial Cells.** Adhesion abilities of the \(K. pneumoniae\) strains were determined following the previously described procedure (44, 45), with some modifications. Three cell lines were used in this experiment, including A549 human alveolar epithelial cells, HT-29 human colorectal epithelial cells, and HT-1376 human urinary bladder epithelial cells. The A549 cells were grown in the presence of 5% CO\(_2\) at 37 °C, on DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS.
Bacterial growth was measured at OD600 to estimate total cell biomass. And biofilm formation was visualized by staining with 0.1% Crystal violet.

Experiments were performed in duplicate for each strain. Bacterial cells were grown under the same conditions used to infect filters, respectively, were used. Images were captured at random at the excitation wavelengths were 488 nm and 50 mg/mL to determine the bacterial burden. The presence of the complement plasmid in the complemented strain Kp3380ΔkpiD_C was tested on day 3 after inoculation by PCR.

In Vivo Murine Models. The role of the Kpi plus system in intestinal colonization and lung infection was assessed using two experimental mouse models. The intestinal colonization of the type strain Kp3380 and the two other groups were infected with the wild-type strain Kp3380 and the two other groups were infected with the Kp3380ΔkpiD and Kp3380ΔkpiD_C strains. Larvae injected with an equivalent volume of sterile PBS were used as controls. The infected and control larvae were incubated at 37 °C in darkness, and death was monitored at 8-h intervals during 5 d. The resulting survival curves were plotted using the Kaplan–Meier method. The presence of the complementation plasmid in the complemented strain Kp3380ΔkpiD_C was tested at 15 h postinfection by PCR. The log-rank (Mantel–Cox) test was used to compare the survival distributions of two groups: The larvae infected with Kp3380 and the larvae infected with Kp3380ΔkpiD.

Quantitative Biofilm Assay. Biofilm formation was quantified following a previously described procedure (46), with some modifications. K. pneumoniae strains were grown on LB broth overnight at 37 °C with shaking. Overnight cultures were diluted 1:100 and inoculated in 48-ml flat-bottom polystyrene microtiter plates (Corning Costar TC-Treated Multiple Well Plates). Samples were incubated at 37 °C for 24 h under static conditions, and biofilm formation was visualized by staining with 0.1% Crystal violet. Bacterial cells were removed by centrifugation (4,000 × g, 15 min), washed twice, and resuspended in PBS (1:1 dilution). The infected monolayers were washed three times with saline solution and lysed in 500 µL of 0.5% sodium deoxycholate (Sigma-Aldrich). Dilutions of the lysates were plated onto LB agar and incubated at 37 °C for 24 h. In order to determine the percentage of bacteria that attached to A549, HT-29 or HT-1376 cells, colony-forming units (CFU) were counted 24 h later. The percentage of attached bacteria was compared with the total number of infecting bacteria.

Animal Research. All experiments were conducted in accordance with regulatory guidelines and standards established by the Animal Ethics Committee of the A Coruña University Hospital Complex (Spain). This committee follows the recommendations of the National Committee for the Replacement, Refinement, and Reduction of Animal Research.

Data Availability. The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. TEM and CLSM images are openly available in Zenodo at https://zenodo.org/record/39062419.axios/2Kkk4.

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The intestinal colonization capacity of the type strain Kp3380 and the two other groups were infected with the wild-type strain Kp3380 and the two other groups were infected with the Kp3380ΔkpiD and Kp3380ΔkpiD_C strains. Larvae injected with an equivalent volume of sterile PBS were used as controls. The infected and control larvae were incubated at 37 °C in darkness, and death was monitored at 8-h intervals during 5 d. The resulting survival curves were plotted using the Kaplan–Meier method. The presence of the complementation plasmid in the complemented strain Kp3380ΔkpiD_C was tested at 15 h postinfection by PCR. The log-rank (Mantel–Cox) test was used to compare the survival distributions of two groups: The larvae infected with Kp3380 and the larvae infected with Kp3380ΔkpiD.
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