Klebsiella pneumoniae as a key trafficker of drug resistance genes from environmental to clinically important bacteria

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Abstract

Klebsiella pneumoniae is an opportunistic bacterial pathogen known for its high frequency and diversity of antimicrobial resistance (AMR) genes. In addition to being a significant clinical problem in its own right, *K. pneumoniae* is the species within which several new AMR genes were first discovered before spreading to other pathogens (e.g. carbapenem-resistance genes KPC, OXA-48 and NDM-1). Whilst *K. pneumoniae*'s contribution to the overall AMR crisis is impossible to quantify, current evidence suggests it has a wider ecological distribution, significantly more varied DNA composition, greater AMR gene diversity and a higher plasmid burden than other Gram negative opportunists. Hence we propose it plays a key role in disseminating AMR genes from environmental microbes to clinically important pathogens.

Short title: Drug resistance gene dissemination by *Klebsiella*

Keywords: *Klebsiella pneumoniae*; horizontal gene transfer; antimicrobial resistance; microbial ecology; plasmids

Antimicrobial resistance in Gram negative opportunistic pathogens

The antimicrobial resistance (AMR) crisis facing hospitals globally is driven by the ESKAPE pathogens (Gram positives *Enterococcus faecium, Staphylococcus aureus*; and Gram negatives *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter*), which are responsible for the majority of infections in hospital patients that are difficult to manage with antimicrobial therapy [1]. Notably the ESKAPE pathogens are environmental or commensal bacteria that cause opportunistic infections in hospitalised or immunocompromised patients, but are generally not pathogenic otherwise. Each of these species has intrinsic resistance to one or more antibiotics, and individual strains have accumulated resistance to many additional drugs [1]. The Gram negative ESKAPE pathogens are considered the greatest threat [2], due to the emergence of strains that are resistant to all or most available antibiotics [3]. Accumulation of AMR in these organisms is primarily due to horizontal gene transfer (HGT) aided by plasmids and mobile genetic elements [1]. The catalogue of known mobile AMR genes subject to HGT amongst Gram negative pathogens numbers in the hundreds [4]. The origins of the AMR genes themselves are environmental bacteria (particularly soil bacteria), assumed to be those which have co-evolved with the relevant antimicrobial producing organisms for millennia [5–7]; however there is typically a lag of several years between the clinical use of a drug and the arrival of relevant mobile AMR genes in human pathogen populations [8]. Hundreds of mobile AMR genes have been found in *K. pneumoniae* [9,10], the species associated with the earliest reports of many AMR genes before their dispersal amongst other clinically relevant Gram negatives. Here we discuss this phenomenon in detail, and then explore what is currently known about *K. pneumoniae* ecology and its genome plasticity, arguing that these characteristics position the species as a key amplifier and spreader of AMR genes from environmental sources to human pathogen populations.

The canary in the coalmine

K. pneumoniae are intrinsically resistant to ampicillin due to the presence of the SHV-1 penicillinase in their chromosome [9,11]. Resistance to additional drugs occasionally arises through chromosomal mutations [12], however most AMR in *K. pneumoniae* results from acquisition of AMR genes via HGT, mainly via large conjugative plasmids [10,12,13]. The accumulation of resistance determinants in a single strain can result in pan-resistant strains that are untreatable with all available antibiotics [3]. The earliest mobile ampicillin resistance genes identified in Gram negative bacterial populations were TEM (present in the first described plasmids in the 1960s), and the *K. pneumoniae* chromosomal SHV-1 gene which was first detected in mobile, plasmid-borne form in other Enterobacteriaceae in 1973 [14,15] (**Fig 1**). Following the introduction of third generation cephalosporins for clinical use in the early 1980s, extended spectrum beta-lactamase (ESBL) genes conferring resistance to these drugs began to be detected and characterised. The earliest forms include ESBL variants of mobile SHV (SHV-2; 1985) [16], TEM (1984) and CMY (1989) [17], which were first identified in *K. pneumoniae* (**Fig 1**) and are now widespread amongst Enterobacteriaceae [18], and in some cases have also spread to *Acinetobacter* [19] and *Pseudomonas* [20]. The most widely dispersed ESBL gene is CTX-M, variants of which were detected in *E. coli* and *K. pneumoniae* in the late

1980s and early 1990s, having been mobilised out of environmental Enterobacteriaceae (*Kluyvera*) [21,22]. CTX-M is now intimately associated with the *E. coli* ST131 pandemic clone [23] and several *K. pneumoniae* clones [12], and is present in diverse plasmid backgrounds, resulting in broad dissemination amongst hospital, human commensal, and animal associated microbial populations [23,24].

The 1990s introduction of carbapenems and fluoroquinolones were met by rapid appearance of associated resistance genes, with *K. pneumoniae* often playing a key role (**Fig 1**). Mobile quinolone resistance genes *qnrA* and *qnrB* were first identified in *K. pneumoniae* [25,26], following mobilisation from marine bacterium *Shewanella* [27], and are now common amongst Enterobacteriaceae plasmids. The *K. pneumoniae* carbapenemase (KPC) appeared in the mid-1990s in the USA and drove the spread of pandemic hospital outbreak clone *K. pneumoniae* ST258, which is now globally disseminated [23]. The KPC gene has been transferred to many different plasmids, is now widely dispersed amongst Enterobacteriaceae and has also found its way into *Pseudomonas* [28] and *Acinetobacter* [29]. The OXA-48 carbapenemase originates from *Shewanella* [30] and was first detected in *K. pneumoniae* in Turkey in 2003 [31]. It was initially associated with hospital outbreaks across Europe and is now reported worldwide [32], although not as widely dispersed as KPC. The NDM-1 metallo-beta-lactamase was first detected in 2008 in *K. pneumoniae* from a patient who had recently travelled to India [33]. The gene was plasmid-borne and shortly thereafter was reported in different *K. pneumoniae* strains isolated from patients with and without recent travel [34]; by 2010 NDM-1 had spread to numerous plasmids and Enterobacteriaceae species, was detected within the chromosome of *E. coli* and *Providencia stuartii* [35], and was spreading amongst *Acinetobacter* and *Pseudomonas* [36,37]. The first mobile colistin resistance gene MCR-1 was reported in China in 2015 in *E. coli* and *K. pneumoniae* [38]; by 2016 it had been detected across five continents, among *Enterobacter* and numerous other species, and in association with over a dozen distinct plasmids [39].

It is impossible to accurately reconstruct the precise flow of genes, plasmids and bacteria involved in the capture of AMR genes from environmental microbes and their dissemination among human-associated bacterial pathogen populations, although *qnrA* and OXA-48 provide compelling examples of AMR gene mobilisation from marine bacteria to *K. pneumoniae*, and onwards to other ESKAPE pathogens. Regardless of precise HGT flow, the dominance of *K. pneumoniae* amongst early clinical reports of new AMR genes is notable, and indicates *K. pneumoniae* to be a prime target for sentinel surveillance of new AMR genes entering Gram negative pathogen populations.

Means and opportunity: genome plasticity, plasmid diversity and ecology

Figure 2A shows the total number of distinct acquired AMR genes and load per strain for all genome sequences of *K. pneumoniae* and fellow Gram negative opportunists (*A. baumannii, P. aeruginosa, E. cloaceae* and *E. coli*) currently in the NCBI Pathogen Genomes portal (>1400 genomes for each species). Over 400 acquired AMR genes are present in the *K. pneumoniae* genomes, double the number in *E. coli* and 50% more than that of the other species (**Fig 2A**), suggesting that *K.*

pneumoniae receives and/or amplifies a wider range of AMR genes from their ultimate source in environmental microbes [6,7]. Below we consider the genomic and ecological characteristics of *K. pneumoniae* in comparison to the other ESKAPE pathogens and *E. coli,* highlighting factors that may enhance exposure to environmental AMR genes and the ability to pass these genes on to other clinically important pathogens.

Ecological range

Most HGT occurs between cells residing in the same habitat [40], hence bacteria adapted to survive in environmental and animal/human-associated microbial communities can be predicted to contribute most to the trafficking of AMR genes between these niches. Reports in the 1970s-80s highlighted the ubiquitous distribution of *K. pneumoniae* among diverse fresh and salt water environments, plants, and soil [41]. *K. pneumoniae* causes infections in cows, horses and other wild and domestic animals [9,42–49]. However as an opportunistic pathogen, it is likely that *K. pneumoniae* is more often a component of the normal animal gut microbiota. In humans the rate of intestinal *K. pneumoniae* colonisation has been estimated at ~6% [50,51], while in dairy cows the rate may be much higher (~44% among herds in New York, USA, [52]). *K. pneumoniae* has also been cultured from the faeces of other agricultural and domestic animals, from the cloacae of birds, and from fish, shellfish, insects and earthworms [43,49,53–57]. It is a common contaminant of animal and plant-based foods, which likely plays a key role in introducing environmental strains into the human gut [49].

Enterobacteriaceae are well known gut colonisers, and all the ESKAPE pathogens can be isolated from environmental sources [58], however systematic comparisons of isolation rates across environmental and animal sources are lacking. We used the IMNGS website [59] to query the growing body of publicly available 16S taxonomic profiling data for the presence of Gram negative opportunists across a wide range of sample types (**Fig 3A**). These data confirm the three Enterobacteriaceae species are frequent commensals of humans and animals, with *E. coli* the most commonly detected; however *K. pneumoniae* and *E. cloaceae* showed equivalent or even greater prevalence amongst plant and environmental samples (7-14%), whereas *E. coli* was significantly less common in these niches (3-4%, p=0.01). Notably these data indicate that *K. pneumoniae* and *E. cloaceae* have similarly broad ecological distributions, but *K. pneumoniae* was more prevalent in human and other animal microbiomes (6.8% vs 3.6%), likely increasing its exposure to antimicrobial use and its contact with other clinically important pathogens, therefore enhancing amplification and onward dissemination of acquired AMR genes.

It is difficult to directly assess movement of individual *K. pneumoniae* strains between niches, however there is evidence that isolates from human, animal and environmental sources do not represent distinct subpopulations [9,43,49,60–62]. Clinically important *K. pneumoniae* lineages have been isolated from specific non-human sources (**Fig 3B**); e.g. ESBL ST15 in cats and dogs [60–62], and hypervirulent ST23 or ST25 in horses, non-human primates and pigs [44,63,64]. Genomic comparisons of *K. pneumoniae* from diverse sources are rare but show little

evidence of segregation between niches: in a global diversity study, 59 bovine isolates were distributed around the species phylogeny comprising mostly human isolates [9]; and a comparison of ESBL isolates from Thai hospitals and a local canal system indicated phylogenetic intermingling [65]. These data and others [49] confirm that at least some strains, including those recognised as globally distributed hospital pathogens [12], can move between and proliferate in multiple niches, providing opportunity for genetic exchange with a broad range of bacterial species (**Fig 3B**).

Genome composition and HGT

K. pneumoniae genomes are highly diverse [9], comprising hundreds of distinct phylogenetic lineages that differ from each other by ~0.5% nucleotide divergence. Individual strains harbour ~2000 'core' (shared) genes, plus a further ~3500 accessory genes that differ between strains and are drawn from a large pool of >30,000 [9]. The ~2000 core genes likely facilitate *K. pneumoniae*'s broad ecological range by providing metabolic and other capabilities enabling survival in a wide range of niches. A substantial proportion of the total pan-genome (core + accessory genes, [66]) is predicted to encode proteins with metabolic functions; 19% associated with carbohydrate metabolism, 18% with other metabolic pathways and 13% with membrane transport [9]. This extensive diversity results in variable metabolic capacity [67], potentially supplementing individual strains with additional ecological range and providing even more opportunities for genetic exchange. Direct comparison of population structures are difficult due to different sampling and analysis strategies [66]; nonetheless it is clear that the other Gram negative opportunists also have many deep branching lineages and large pan-genomes [9,68–71]. Coding capacity and genome size are easy to compare using public genome data (**Fig 2C**): *K. pneumoniae* has a significantly larger genome than the other Enterobacteriaceae species considered here (mean 5.7 Mbp, 5455 protein coding genes, vs 5.1 Mbp/4915 genes in *E. coli* and 5.0 Mbp/4680 genes in *E. cloacae*; $p<1x10^{-15}$ using two-sided t-test), which may help equip *K. pneumoniae* for survival in a broader range of niches.

DNA base composition varies widely between taxa, and can be used as a signature of bacterial species [72]. The mean G+C content of *K. pneumoniae* core genes is 58%, whereas that of accessory genes ranges from 20% to >70%, suggesting they originate from a taxonomically diverse array of donors [9]. **Figure 2D** shows that genes annotated in complete genomes of *K. pneumoniae* display significantly more variability in their G+C content than those of the other species considered here, with 50% greater variance in G+C content than *E. coli* and *E. cloaceae* ($p<1x10^{-15}$ using F-test or the non-parametric Fligner-Killeen test) and more than double the variance of *P. aeruginosa* and *A. baumannii*. This suggests *K. pneumoniae* receives DNA from a wider diversity of HGT partners; indeed lowest common ancestor analysis of *K. pneumoniae* accessory genes has implicated >20 distinct genera as DNA donors, including numerous other members of the Enterobacteriaceae but also diverse groups such as *Acinetobacter*, *Burkholderia*, *Streptomyces, Vibrio*, *Xanthomonas* and *Xyella* [9]. More direct evidence for *K. pneumoniae* engaging in inter-species HGT can be found in recent genomic comparisons of carbapenem-resistant

Enterobacteriaciae in hospitals, which captured identical or highly similar carbapenemase encoding plasmids and/or transposons from *K. pneumoniae* and other species originating from the same ward and/or patient (*E. coli, E. cloacae, Enterobacter asburiae, Citrobacter freundii, Klebsiella oxytoca, Raoultella ornothinolytica*) [73–75].

Plasmid load

The vast majority of AMR genes in *K. pneumoniae* are plasmid-borne [10,12], hence the ability to amplify and spread AMR genes across ecological niches is likely linked to plasmid-permissive traits. Highly diverse environmental microbial communities, especially soils, are considered hotspots for gene transfer [76], and Enterobacteriaceae have been identified as a component of the "super-permissive community" that supports the spread of plasmids across diverse soil communities [77]. The specific role of *K. pneumoniae* in such activities remains to be explored, however the species has been associated with hundreds of distinct plasmids spanning many plasmid replicon types [9,10,13,78], which suggests it acts as a recipient for plasmids originating from a wide array of HGT donors. The median number of plasmids per complete *K. pneumoniae* genome currently in NCBI GenBank is three (interquartile range, 2-5; range 0-10), significantly higher than that for the other species of interest ($p<1x10^{-5}$, see **Fig 2B**). This is consistent with numerous reports of *K. pneumoniae* strains carrying multiple AMR plasmids; e.g. the ST11 reference genome, HS11286, harbours six plasmids (1.3-123 kbp in size), three of which carry AMR genes [79]; and there are many examples in other lineages [10,80,81].

Its elevated plasmid load (**Fig 2B**) suggests *K. pneumoniae* is particularly permissive for plasmids, meaning it may be more likely to capture plasmid-borne material from diverse donors in varied niches, and to hold on to this material long enough to transmit it to new recipients in human and animal-associated niches (**Fig 4**). This enhanced permissiveness may reflect a comparatively lower fitness burden of plasmid carriage in *K. pneumoniae*; a hypothesis supported by a small number of studies showing lower fitness costs for specific AMR plasmids in *K. pneumoniae* vs *E. coli in vitro* [82,83], and reports of long-term plasmid maintenance in *K. pneumoniae in vivo* [64,73,83]. Plasmid-host interactions are complex, and there are many reported examples of specific adaptations of hosts to plasmids (and *vice versa*) [84,85]. It is has recently been shown that some chromosomal adaptations (such as helicase and RNA polymerase mutations) can increase general plasmid permissiveness of a host bacterium [85]. Intriguingly, it is clear that AMR genes and plasmids are not randomly distributed amongst *K. pneumoniae* lineages [9,10], suggesting that there may be significant variation in plasmid permissiveness between lineages. Related species and strains can vary substantially in their ability to act as plasmid donors [77,86], however variation in plasmid-donor potential between *K. pneumoniae* and other bacteria, or between strains of *K. pneumoniae*, remains to be investigated.

Conclusions

K. pneumoniae have the means and opportunity to capture plasmids from environmental microbial populations; to survive within and move between multiple environmental and animal-associated niches; to maintain AMR plasmids for prolonged periods; and to pass plasmids on to other clinically important Gram negative bacteria (**Fig 4**). Whilst the contribution of *K. pneumoniae* to the AMR crisis is impossible to quantify, the available evidence suggests it is unique amongst the Gram negative ESKAPE pathogens and *E. coli* in a few key respects including its high diversity of acquired AMR genes, high plasmid load, wide variability of G+C content reflecting diverse HGT partners and broad ecological range – whilst systematic studies of comparative ecology are lacking, the available 16S data suggests *K. pneumoniae* is equally likely to be found living in human, animal and environmental niches. Combined these factors may position *K. pneumoniae* as a key amplifier and spreader of clinically important AMR genes. Better understanding and monitoring of this highway of AMR gene transfer could potentially help limit the spread of AMR and prolong the life of new antibiotics.

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Figures

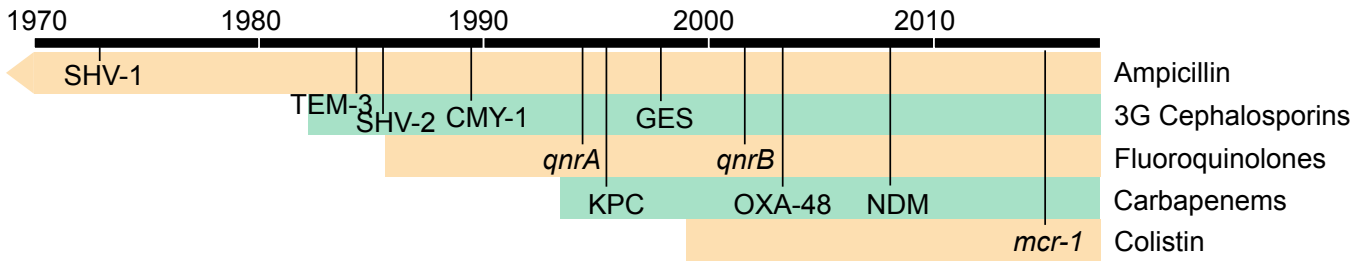

Figure 1. Timeline of mobile AMR genes first detected in *Klebsiella pneumoniae*. Shading indicates the period since which isolates of *K. pneumoniae* resistant to each drug class have been reported (regardless of mechanism). Selected mobile AMR genes that were first detected in *K. pneumoniae* are labelled on the timeline, within the row corresponding to the relevant class; all have since been reported in clinically important Enterobacteriaceae and other Gram negative bacteria. Note ampicillin resistance is intrinsic to *K. pneumoniae* due to the chromosomal beta-lactamase gene SHV-1, and this gene was shown to be mobilised by plasmids in *E. coli* and *K. pneumoniae* in the 1970s. The other genes shown did not originate in *K. pneumoniae*, but they were first detected in mobile form (i.e. within mobile genetic elements on plasmids) in *K. pneumoniae* isolates, as detailed in the "The canary in the coalmine" section.

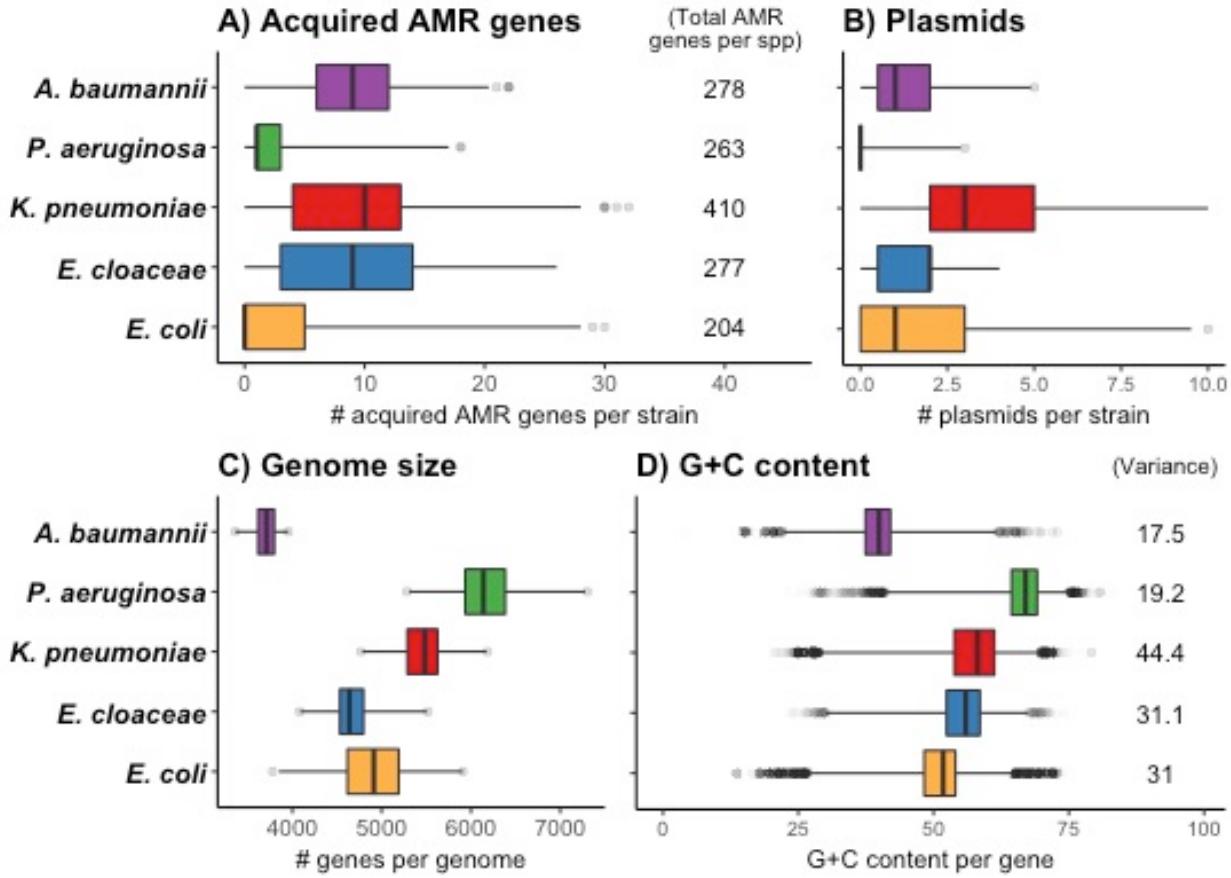

Figure 2. Genome characteristics and burden of acquired AMR genes and plasmids in Gram negative ESKAPE pathogens and *E. coli*. (A) Acquired AMR gene count per genome, extracted from the NCBI Pathogen Detection portal (>1400 genomes per species). The following intrinsic genes were excluded: *fosA*, *catB*, *aph(3')-IIb* in *P. aeruginosa*; *fosA, oqxA*, *oqxB* in *K. pneumoniae*; *fosA*, *catA*, *oqxB* in *E. cloacae*; *bla*EC in *E. coli*. **(B-C)** Number of plasmid replicons and protein-coding genes annotated in all complete genome sequences in NCBI GenBank (484 *E. coli*, 31 *E. cloaceae*, 198 *K. pneumoniae,* 114 *P. aeruginosa*, 95 *A. baumannii*). **(D)** G+C content per gene annotated in the same set of complete genomes. All data was downloaded from NCBI on March 19, 2018; code to generate plots is available in FigShare (doi: 10.4225/49/5ac3670f83717).

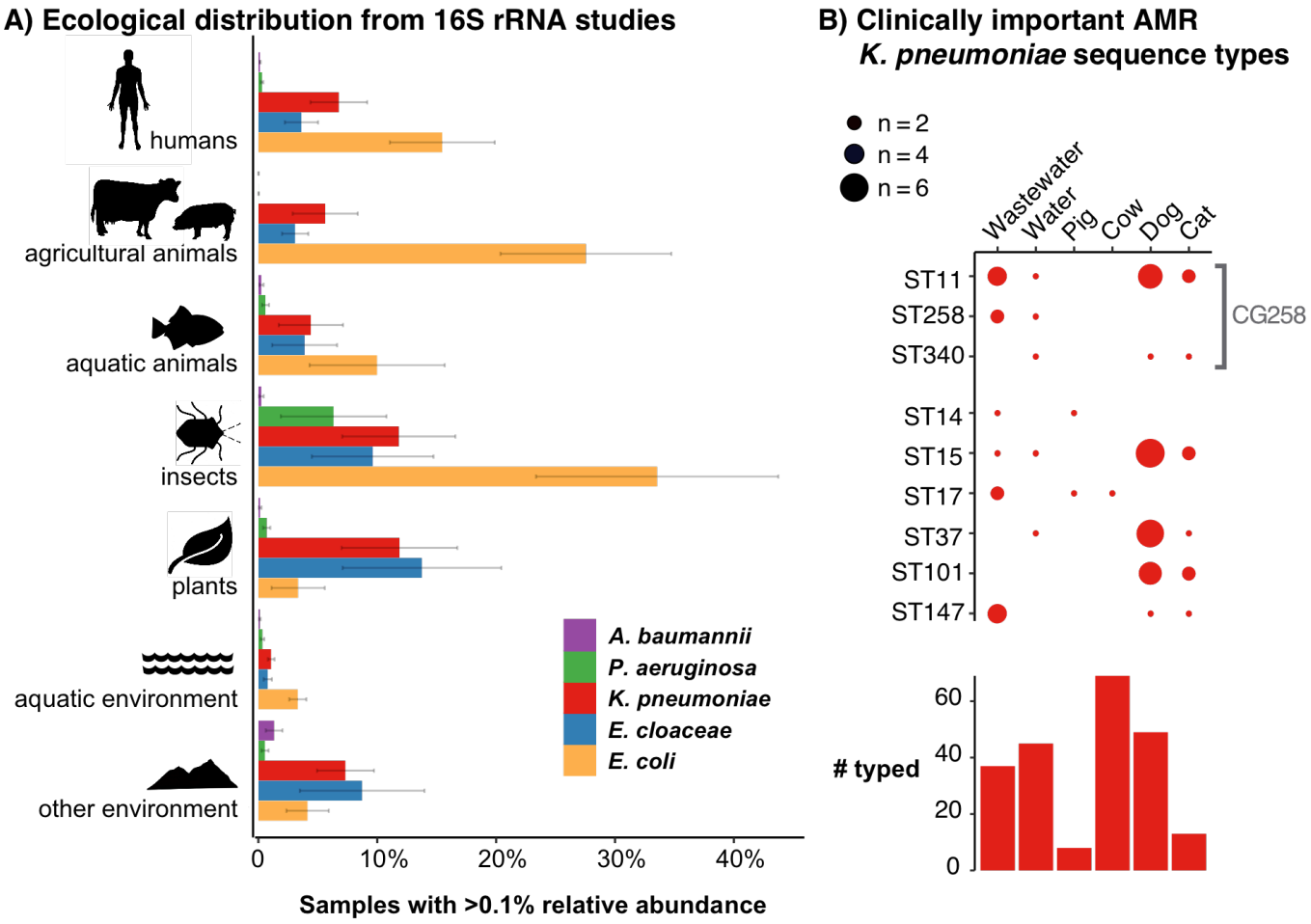

Figure 3. Ecological distribution of species and sequence types based on public sequence data. (A) The IMNGS database (imngs.org, [59]) was queried in March 2018 using one 16S rRNA sequence each from *Klebsiella pneumoniae* (NJST258_1), *Acinetobacter baumannii* (A1), *Pseudomonas aeruginosa* (PA01), *Enterobacter cloaceae* (ATCC 13047) and *E. coli* (K-12), for hits with ≥99% identity and ≥200 bp. Bars indicate mean frequency of detection (defined as relative abundance >0.1%) for each species in category descriptors containing ≥100 samples; error bars indicate standard error of the mean. IMNGS output and code to generate plots is available in FigShare (doi: 10.4225/49/5ac3670f83717). **(B)** Top: globally distributed AMR STs reported from at least 2 non-human sources; CG258 = KPC-associated clonal group 258. Below: bar heights indicate number of isolates for which STs were reported in the literature (March 2018) and/or inferred from published genome data. Data represent published STs from reports identified by PubMED search for "Klebsiella pneumoniae" AND "ST*" AND one of "cat", "feline", "dog", "canine", "cow", "bovine", "pig", "porcine", "water", "aquatic" ("horse" and "equine" were also searched but yielded no reports of globally distributed AMR STs); additional STs were compiled from two genomic studies which had each reported >10 isolates from non-human sources [9,65].

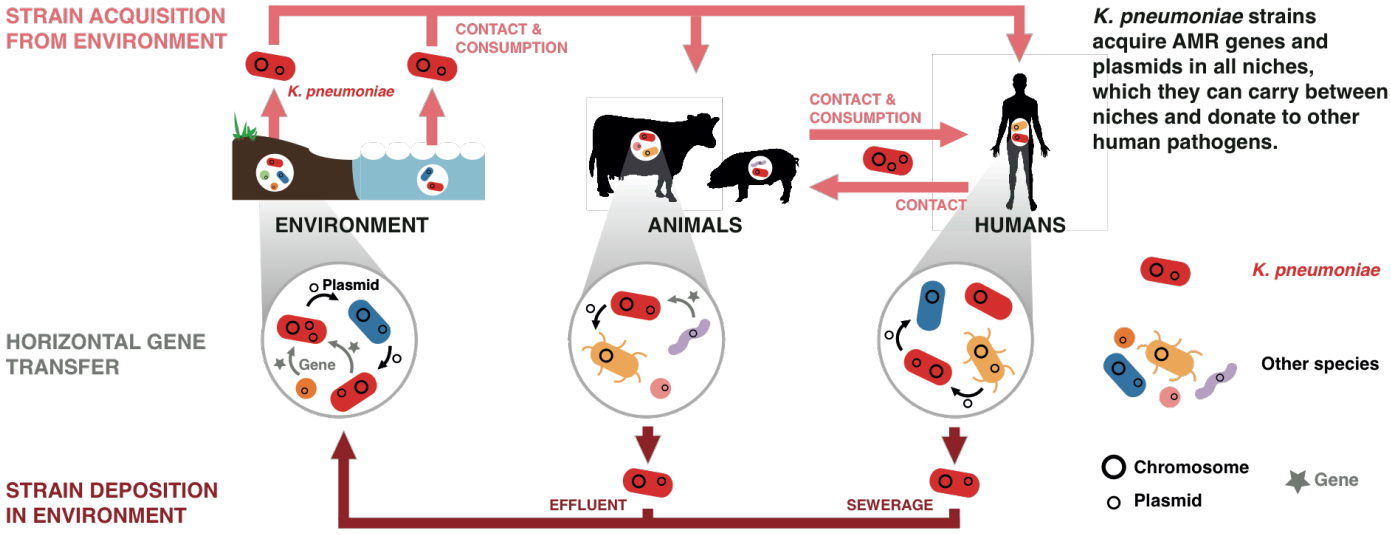

Figure 4. Model for AMR gene and plasmid trafficking by *K. pneumoniae*. Individual *K. pneumoniae* strains can move between niches in the environment, human and/or animal hosts, carrying with them acquired AMR genes and/or plasmids. Strains can move from the environment to human/animal hosts via contact or consumption of contaminated water sources or plant matter; between human and animal hosts via contact or consumption; and from hosts back to the environment via effluent or sewerage. *K. pneumoniae* strains can receive or donate plasmids via HGT with a diverse array of donor species in any of these niches, providing a pathway for transfer of AMR genes from environmental microbes to human pathogens.