

Genomic Characterization of an Emerging *Enterobacteriaceae* Species for the First Case of Human Infection in Cooperation with a Typical Pathogen

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Abstract

Opportunistic pathogens are important for clinical practice as they often cause antibiotic resistant infections. However, little is documented for many emerging opportunistic pathogens and their biological characteristics. Here, we isolated a novel species of extended-spectrum β -lactamase-producing *Enterobacteriaceae* from a patient of biliary tract infection. The isolate grows very slowly but confers strong protection for the co-infected cephalosporin-sensitive *Klebsiella pneumoniae*. As the initial laboratory testing failed to identify the taxonomy of the strain, great perplexity was caused in the etiological diagnosis, and anti-infection treatment for the patient. Rigorous sequencing efforts achieved the complete genome sequence of the isolate which we designated as AF18. AF18 is phylogenetically close to a few strains respectively isolated from soil, clinical sewage, and patients, forming a novel species together, while the taxonomic nomenclature of which is still under discussion. And this is the first report of human infection of this novel species. As its relatives, AF18 harbors many genes related to cell mobility, various genes adaptive to both wild environment and animal host, tens of genetic mobile elements, and a plasmid bearing *bla*_{CTX-M-3} gene, indicating its ability to disseminate antimicrobial resistant genes from wild to patients. Transcriptome sequencing identified two sRNAs that critically regulate the growth rate of AF18 which could serve as targets for novel antimicrobial strategies. These findings imply that AF18 and its species are not only infection-relevant but also potential disseminators in transferring antibiotic determinants, which highlights the need for continuous monitoring for this novel species and efforts to develop controlling strategies.

Introduction

Antimicrobial resistance (AMR) is an increasingly global health threat that contributes to tens of thousands of deaths per year (J, 2014). Increased and often unrestricted antibiotic use in the clinical and farming settings is blamed for this issue. Growing surveillances based on genomic sequencing of microbes from nature environment, human settlements, and clinical settings have been conducted worldwide to investigate the evolution and transfer of antibiotic resistance genes (ARGs) (Woolhouse et al., 2015; Hassell et al., 2019; Mourkas et al., 2019). In recent years, the eco-evolutionary feedback loops between ecological and evolutionary dynamics has been increasingly recognized, where spillover of antibiotic use to natural and semi-natural environments may have profound implications on the distribution of ARGs in natural bacterial populations which serve as environmental reservoirs of resistance determinants (Woolhouse and Ward, 2013; Hiltunen et al., 2017). However, how resistance evolves, and how ARGs are maintained and dispersed back to clinical settings is poorly understood. Understanding the dynamics of the continuous feedback loops from clinical to nature and back may prove critical for preventing and controlling the problem of antibiotic resistance.

The rapidly developing sequencing technology enables more and more emerging opportunistic pathogens to be identified and taxonomically classified based on their genomic information (Romano-Bertrand et al., 2016; Schurch and van Schaik, 2017; Ekundayo and Okoh, 2018). Naturally, opportunistic pathogens inhabit in wild and are occasionally resistant to common antibiotics. Among these previously

unknown pathogens, many are species of the Enterobacteriaceae family (Shin et al., 2012; Hunter and Bean, 2013). Meanwhile, Enterobacteriaceae species associated to human are often residents in human and animal guts, but in certain conditions can be opportunistic pathogens that cause infections (Taylor et al., 2001). These species often have other animal hosts, or they can be found in the wider environment, such as soil and sewage, or both (Mather et al., 2013). Enterobacteriaceae species (including *E. coli*, *Klebsiella*, and *Enterobacter*) are also famous for their antibiotic resistance and regarded as one of the most dangerous pathogens that they can efficiently acquire various ARGs through efficient plasmid transmission (Iredell et al., 2016). The ability of these species in transferring between habitats and transferring ARGs potentiates them to be important mediators in the eco-evolutionary feedback loops that disperse ARGs back to clinical settings from wild environment. The taxonomy of Enterobacteriaceae is complex that contains 28 genera and over 75 species (Adeolu et al., 2016), while novel species are continuously discovered. To fully recognize and characterize Enterobacteriaceae species, especially those of emerging opportunistic pathogens, is critical for understanding the dynamics of the evolution of AMR.

Here, from a patient of biliary infection, we isolated a novel strain of unknown taxonomy accompanying an infectious *Klebsiella pneumoniae* strain. The isolate grew slowly but provided drug-resistance to its companion by carrying a $bla_{CTX-M-3}$ resistant gene. The co-infection brought perplexity in both diagnosis and treatment of the patients. Complete genome sequencing based on both SMRT (single-molecular real-time sequencing) and Illumina platform achieved a high-quality genome of this resistant strain, which suggested it as a strain in Enterobacteriaceae but of an undefined novel species. Together with a transcriptome sequencing, we are able to take a deep insight into the genomic characteristics of the rare pathogen and regulation mechanisms of how it adapts to multiple habitats and associates to ARG transfer.

Materials And Methods

Biological Characterization of Strain AF18

Colony of AF18 was applied to assay using the VITEK-II automated bacterial identification system and the API20E *Enterobacter* biochemical identification system (Biomérieux, France). The TOF-MS -based identification was conducted with a MicroFlex LT mass spectrometer (Bruker Daltonik) to obtain the protein profile of AF18, which was further analyzed using MALDI Biotyper software (Bruker Daltonik). The morphology of the bacteria was observed under a Hitachi S-3400N scanning electron microscope with a standard procedure. Briefly, a colony of AF18 was fixed with 2.5% glutaraldehyde followed by 1% osmium acid for 2 hours each, and then gradient dehydrated with ethanol and dried in a desiccator. The prepared sample was then observed under the EM after gold coating. Flagellum on AF18 cells was observed under JEM-1230 transmission electron microscope after stained with 1% uranyl acetate.

Antimicrobial susceptibility of strain AF18 and *K. pneumoniae* was investigated by broth microdilution using the E-test (bioMérieux) according to manufacturers' instructions.

Genome Sequencing

AF18 cells were harvested from its overnight culture by centrifugation, and DNA was extracted with QIAamp DNA Mini Kit (Qiagen, Cat No: 51304) following manufacturer's instruction. The extracted DNA sample was assayed with NanoDrop spectrophotometer for quantification and then sent to Beijing Novogene Bioinformatics Technology Co., LTD for whole genome sequencing. The genome of AF18 was both sequenced with the single molecule real-time sequencing (SMRT) technology from Pacbio using a PacBio RSII sequencer (insert size was approximately 10 kb) for one cell, and sequenced with the short-reads HiSeq 2000 platform from Illumina (100bp × 2) for 3G raw reads. The obtained raw SMRT reads were analyzed and de novo assembled using SMRT Analysis 2.3.0 software. Error correction of tentative complete circular sequences was performed using Pilon version 1.18 with Illumina short reads.

Genome Annotation

GeneMarks (version 4.17) (Besemer et al., 2001) was used to predict protein-coding genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), NCBI-NR and Gene Ontology (GO) databases were used to annotate the function of predicted genes (Kanehisa and Goto, 2000; Tatusov et al., 2000; Harris et al., 2004). Virulent genes were reliably identified by BLAST in the Virulence Factors Database (VFDB) with identity > 40%, and antibiotic-resistant genes were identified using RGI from the Comprehensive Antibiotic Resistance Database (CARD) with "perfect and strict hits" (Jia et al., 2017; Liu et al., 2019).. Circos software (Version 0.64) was used to plot the circular map of genome (Krzywinski et al., 2009). Plasmid replicon typing was performed using the curated PlasmidFinder database at the CGE website (Carattoli and Hasman, 2020).

Phylogenetic Analysis

We used Mash (Ondov et al., 2016) to compare the AF18 genome sequence to genome assembly database from NCBI, and picked 33 non-redundant species with identity score > 75%. Then, we used kSNP3 to identify core genome SNPs of each pair of the 34 genome sequences with an optimal k-mer size of 21 (determined by Kchooser) (Gardner et al., 2015). These core SNPs were used to build a maximum likelihood tree by FastTreeML (Price et al., 2010), and iTOL was used to exhibit the phylogenetic tree (Letunic and Bork, 2016). We used fastANI to calculate the pairwise ANI of the 34 genome sequences (Jain et al., 2018).

Comparative Analysis Of Paf18_2

We blast pAF18_2 against the nr/nt database, and picked top 10 non-redundant plasmids according to the query coverage and alignment score (Johnson et al., 2008). We then performed BLASTN for pAF18_2 against the other 10 plasmids with E-value < e-50 (Camacho et al., 2009), and generated the comparative map using CGView (Stothard and Wishart, 2005).

Drug-resistant Plasmid Elimination Test

A single colony of AF18 was inoculated into LB medium without antibiotics and cultured for 24 hours at 37 °C. Then the culture was 1:1000 diluted and re-inoculated in LB medium for another 24 hours. The

procedure was repeated while an aliquot was collected and spread on LB agar for each round of re-inoculation. Colonies grown on the LB agar were randomly selected and tested for the presence of $\text{bla}_{\text{CTX-M-3}}$ gene by PCR. The PCR negative colonies, which might have lost their resistant plasmid pAF18-2, were functionally confirmed by inoculation in LB medium both with and without ceftriaxone (20 $\mu\text{g}/\text{ml}$). The strains that lost their ability to survive in the ceftriaxone-containing medium were believed that have depleted the resistant plasmid, and one of them was preserved and named as AF18-NC.

Growth Rate Measurement

Overnight cultures of the *K. pneumonia* strain, AF18, and AF18-NC were sampled and diluted to O.D.=0.10. and then cultured at 37 °C for 24 hour. The turbidity (O.D. value) of the cultures was measured by using a bacterial turbidimeter at 1 hour interval for 24 hours. Proportion of AF18 and the *K. pneumonia* strain in co-cultured samples was determined by colony counting on plates of MacConkey agar.

Transcriptome Sequencing

AF18 and AF18-NC cells were harvest from its overnight culture by centrifuge, and total RNA of both strains were extracted with QIAGEN RNeasy Plus Mini Kit (Qiagen, Cat No.74134) following manufacturer's instruction. The extracted RNA samples were assayed with NanoDrop spectrophotometer for quantification and then sent to Beijing Novogene Co., LTD for transcriptome sequencing. After deletion of rRNA from samples, transcriptome was sequenced on Hiseq 2000 platform with an intended depth of 1G of raw 100bp \times 2 reads. Low quality reads and adaptor sequence were then removed. Using the whole genome of AF18 as the reference genome, the gene expression level for each transcript was estimated by calculating the FPKM (Per Kilobase of transcript sequence per Millions base pairs sequenced) value of each transcript.

Differential Gene Expression Analysis

The read count data of each transcript was first normalized using DEseq (Anders and Huber, 2012). According to the binomial distribution model, hypothesis testing was performed on each transcript between the AF18 strain and the AF18-NC strain, and confirmed by multiple hypothesis tests.

Functions Of Differentially Expressed Genes

Functions of the differentially expressed genes between AF18 and AF18-NC were annotated with GO database (Harris et al., 2004), and the probability of enrichment for each cluster was calculated by using the Goseq algorithm based on the Wallenius non-central hyper-geometric distribution (Young et al., 2012). Clusters with corrected p value < 0.05 was regarded as significantly enriched.

sRNA Analysis

Rockhopper software was used to search new intergenic transcripts (McClure et al., 2013; Tjaden, 2015), and those transcripts did not hit to the NCBI-NR protein database by BLASTx were considered as candidates for non-coding sRNA. The sRNAs with two-fold increased/decreased FPKM value were regarded as up-/down-regulated. Secondary structure of candidate sRNAs were predicted using RNAfold software and their target gene were predicted by using IntaRNA (Hofacker et al., 1994; Mann et al., 2017).

Results

Biological Identification of the Strain AF18

A patient of obstructive jaundice who suffered an infection two days after the percutaneous transhepatic cholangial drainage (PTCD) surgery was admitted to our hospital. From the bile sample of the patient, two types of colonies were isolated after serial dilutions and isolations on MacConkey agar plates. One type was mucous, entirely pink, and of 4–5 mm in diameter, which was finally identified as a *K. pneumonia* clone sensitive to common antibiotics (Table 1); the other type was small colonies of red center, clear and transparent edge, and of 2–3 mm in diameter (Fig. 1A). The bacteria of the small colonies seems prone to adhere to the cells of *K. pneumonia* and were not able to be isolated until extensive dilutions. The taxonomy of the small colonies was not immediately identified by the microbiological laboratory in the hospital and we designated it as strain AF18. AF18 exhibited remarkably resistance to most β -lactam antibiotics in antimicrobial susceptibility testing (Table 1). As the infection was rather intractable and finally cured by intravenous amikacin, the final diagnosis for the patient was a co-infection caused by a sensitive *K. pneumonia* strain and a multidrug resistant strain of unknown species.

Table 1
The antibiotic resistance profile of AF18 and *K. pneumoniae* isolate.

Drug	Antibiotic susceptibility			
	AF18		K. pneumoniae strain	
	MIC ($\mu\text{g/ml}$)	Phenotype	MIC ($\mu\text{g/ml}$)	Phenotype
Ampicillin	≥ 32	R	16	I
Ampicillin/sulbactam	≥ 32	R	4	S
Piperacillin	≥ 128	R	≤ 4	S
Piperacillin/tazobactam	≥ 128	R	≤ 4	S
Cefazolin	≥ 64	R	≤ 4	S
Cefuroxime	≥ 64	R	≤ 1	S
Cefuroxime axetil	≥ 64	R	≤ 1	S
Cefotetan	≤ 4	S	≤ 4	S
Ceftazidime	16	R	≤ 1	S
Ceftriaxone	≥ 64	R	≤ 1	S
Cefepime	≥ 64	R	≤ 1	S
Aztreonam	≥ 64	R	≤ 1	S
Imipenem	≤ 1	S	≤ 1	S
Meropenem	≤ 0.25	S	≤ 0.25	S
Amikacin	≤ 2	S	≤ 2	S
Gentamicin	≤ 1	S	≤ 1	S
Tobramycin	2	S	≤ 1	S
Ciprofloxacin	2	I	≤ 0.25	S
Levofloxacin	1	S	≤ 0.25	S
Nitrofurantoin	256	R	≤ 16	S
Trimethoprim/sulfam	≤ 20	S	≤ 20	S

Microscope observation showed that AF18 was a Gram-negative bacillus (Fig. 1B), and its cells were surrounded by flagella under transmission electron microscope (Fig. 1C). Scanning electron microscope confirmed the tubular shape of AF18 and a smooth surface with no polysaccharide particle (Fig. 1D), in

line with the mucus-free characteristics of its colony. VITEK-II in hospital laboratory did not result in any bacterial species identical to the biochemical properties of AF18 (Table S1), whereas API20E biochemical identification system suggested AF18 as *Pantoea* sp. but with low reliability. The mass spectrometry which scans the protein profile of samples did not identify the species of AF18 either.

Complete Genome of Enterobacteriaceae bacterium AF18

To determine the taxonomy and genetic features of AF18, we performed whole genome sequencing on both platforms of short-reads Illumina Hiseq and long-reads PacBio sequencer and achieved a high-quality completed genome sequence of AF18 which possesses circulated chromosome and two plasmids (Table 2, Fig. S1).

Table 2
Overview of genome information for AF18

Replicon	Nucleotide length (bp)	Coding Genes	GC%	Inc type	Antimicrobial resistance genes	GenBank ID
Chromosome	5,676,372	5651	53.06	NA	ksgA	CP025982
pAF18_1	140,420	181	51.14	IncFII	tetC	CP025983
pAF18_2	42,923	53	51.28	IncN	qnrS, bla _{CTX-M-3} and dfrA	CP025984

By using Mash (Ondov et al., 2016) to search the publicly available bacterial genomes and drafts with a cutoff of mutation distance < 0.25, we identified 33 non-redundant close relatives of AF18, all of which were in the Enterobacteriaceae family. The average nucleotide identity (ANI) matrix of the 34 strains (Fig. 2A) shows that the closest five with identity > 98.5% (i.e. regarded as strains of the same species) are nominated as [*Kluyvera*] *intestini*, *Matakosakonia* sp., *Enterobacter* sp. (two strains), and just Enterobacteriaceae bacterium, respectively, indicating that the nomenclature of this novel species is still under discussion due to very limited documentation (Alnajjar and Gupta, 2017). The first report of the novel species was in 2016 when [*Kluyvera*] *intestini* str. GT-16 was isolated from the stomach of a patient with gastric cancer (Tetz and Tetz, 2016), and in the following years, strains of this species were emergingly discovered (Sekizuka et al., 2018; Weingarten et al., 2018). Of note, AF18 is the first clear report of human infection of this novel species, as [*Kluyvera*] *intestine* GT-16 and *Matakosakonia* sp. MRY16_398 were more likely a common resident in the gastrointestinal tract or a by-stander of the diverticulitis. Although the first strain of [*Kluyvera*] *intestini* str. GT-16 had been assigned to the genus *Kluyvera*, the ANI of strains in this novel species to typical *Kluyvera* spp are less than 80.8%, even farther than the distance to other genus, such as *Kasokonia* (ANI, 82.3%), and typical *Enterobacter* spp. (ANI, 81%), suggesting that AF18 and its species is not a typical *Kluyvera* species or should not be included in this genus. Phylogenetic relationship of these relatives was further inferred with core genome SNPs

(Fig. 2B) which confirmed the relationships inferred from the ANI matrix and indicated the novel species including AF18 possibly stands for another genus than *Kluyvera*. Herein, we temporarily nominated our strain as Enterobacteriaceae bacterium AF18 as the nomenclature of its species even genus name is still undefined.

The chromosome of AF18 possesses 5651 protein-coding genes which functions facilitate the survival and adaptation of AF18 in various habits (Table S2). For example, motility-related genes, including a complete flagellar gene cluster that encodes all components of flagellar, *csg* gene cluster that encodes curli assembly proteins to mediate adhesion, and other genes of *ompA*, *pilRT*, *ibeB*, *icaA*, *htpB* and *fimB*, together confer the ability of adhesion, invasion, chemotaxis, and escape to the host strain. Genes of the *hcp-clp* and *mprAB* system are powerful in implementing persistence status which endows resistance to many environmental stresses including all kinds of antibiotics. Efflux pump genes which confer resistance to macrolides, quinolones and aminoglycosides were also identified. Meanwhile, the AF18 genome possesses 20 genomic islands, 11 prophages and five CRISPR sequences (Table S3), indicating active transfer of stress-adaptive genes by these genetic mobile elements and bacteriophages of this species. More importantly, markers of bacteria inhabited in soil, including a complete nitrogen fixation gene cluster and *ksgA*— a pesticide-resistant gene, were found in AF18 genome which suggests that AF18 is adaptive to dwell in nature environment or a wider range of habits. And the mobility of this strain potentiates it to shuttle between various habits.

Analysis of conserved genes in plasmids shows that most of the antibiotic-resistant genes of AF18, including *qnrS*, *dfrA* and *bla_{CTX-M-3}*, are carried by the smaller plasmid pAF18_2 (Fig. 3) which is responsible for the antibiotic resistance profile of AF18. Sequence alignment shows that pAF18_2 are similar to many plasmids from host of other Enterobacteriaceae species, such as *E. coli*, *K. pneumoniae*, and *C. freundii*, and they contain identical replication origin, replication and transcription system, plasmid partition system, and a partial gene cluster responsible for plasmid conjugation, which indicates that the plasmid might be compatible with all these Enterobacteriaceae host species. Besides, these plasmids share a common anti-restriction system that ensures they would not be destroyed by the restriction-modified system in other host strains. Specifically, the pAF18_2 contains an active transposase system with complete IS elements which had acquired the *bla_{CTX-M-3}* gene and an arsenical resistant system. Many other DNA manipulating enzymes such as integrase and DNA invertase were also identified in the plasmid, all of which facilitate the plasmid in efficiently acquiring and transferring antibiotic-resistance genes and other stress-adaptive genes among Enterobacteriaceae strains.

Growth of AF18 in Co-cultures and Its Transcriptional Regulation

To disentangle the respective contribution of AF18 and the sensitive *K. pneumoniae* in the co-infection, we co-cultivated the two strain in various concentration of ceftriaxone, and found that addition of 1% AF18 was able to elevated the MIC from 0.125 µg/ml of pure *K. pneumoniae* culture to 64 µg/ml. Furthermore, when spread the co-culture onto the MacConkey agar containing ceftriaxone, the sensitive *K. pneumoniae*

colonies were able to withstand 8 µg/ml ceftriaxone (Fig. 4A), indicating a strong protective effect of AF18 to the co-infected *K. pneumoniae*.

Although important in the co-infection for antibiotic-resistance, AF18 only took less than 1% in the initial sample. Even when equally input, the proportion of AF18 decreased to 1% of the co-culture if without antibiotic pressure (Fig. 4B). It seems that AF18 may be less aggressive and its growth rate is much slower than the co-inhabited *K. pneumoniae*. It has been reported that bearing plasmid may slow down growth rate due to the cellular cost caused by the addition of plasmid (Bouma and Lenski, 1988), and thus we generate a new strain—AF18-NC by deleting the resistant plasmid of AF18. Then we measured the independent growth curve of the three strains—*K. pneumoniae*, AF18, and AF18-NC, respectively (Fig. 4C). As expected, AF18-NC did grow faster than its mother strain AF18 as relieved from the plasmid-caused cellular cost. However, the growth rate of AF18-NC was still much slower than that of *K. pneumoniae*, suggesting that slow growth is an inherent property of the novel species.

Next, we analyzed the genes involved in regulation of growth rate by a comparison between the transcriptomes of AF18 and AF18-NC. A total of 3,309 genes of chromosomal coding genes were differentially expressed in significance, with 1675 upregulated and 1634 downregulated in AF18 (Fig. 4D). Functional cluster analysis with GO (Gene Ontology) database showed that most of differentially expressed genes were in the categories of transcriptional regulation, biosynthesis regulation, metabolic process regulation, signal transduction, DNA binding, and signal sensing. Analysis of the non-coding sRNA expression profile identified a total of 15 sRNAs differentially expressed between AF18 and AF18-NC. Interestingly, two of the down regulated sRNAs in AF18, sRNA00063 and sRNA00291 (Fig. S2) shared 98% of their predicted target genes which took up 56% of the above-mentioned differentially expressed coding genes, suggesting their key roles in promoting growth. This result indicated the importance of the two sRNAs in globe regulation of growth rate, and consequently the contribution and competition of the host AF18 in co-infections.

Discussion

In this study, we reported a case of co-infection caused by a typical pathogen and a rare opportunistic pathogen with taxonomical nomenclature undefined. In the pathogenic consortium of the co-infection, the dominant *K. pneumoniae* strain is virulent enough to cause an aggressive infection, while the AF18 strain, although taking a very small proportion, provides strong protection for the entire pathogenic consortium against antibiotic damage. The co-operation between the *K. pneumoniae* and AF18 makes the infectious situation more complicated and difficult in term of therapies than infections caused by either of them. Meanwhile, as the strain AF18 only took a minor proportion and a close adhesion to co-infected *K. pneumoniae*, it was prone to be concealed by the dominant *K. pneumoniae* and hard to be detected and isolated, which led to inaccurate etiological diagnosis and improper anti-infective treatment at first admission. As AF18 and other strains of the same species are rare opportunistic pathogen with little documentation, and conventional testing for bacterial identification are not always correct for such novel

species, as shown in this study, WGS comprised a straightforward approach for accurate taxonomy identification.

Sequencing strategy combining both long- and short-read platforms makes it easy to obtain high-quality complete genome including plasmids, which will be helpful for overall characterization of novel species and deep insight into the functions of the genes they harbor. Phylogenetic analysis with the whole genome of AF18 definitely assigned it to Enterobacteriaceae family. Except AF18 which cause a co-infection with a typical pathogen, two of the other strains of this species ([*Kluyvera*] *intestini* str. GT-16 (Tetz et al., 2017), and *Metakosakonia* sp. MRY16_398 (Sekizuka et al., 2018)) were both isolated from patients in Japan, while the other three strains (*Enterobacter* sp. NFR05, *Enterobacter* sp. Bisph2, and Enterobacteriaceae bacterium ENNIH2) were isolated from rhizoplane (China), sandy soil (Algeria) (Benslama and Boulahrouf, 2016), and hospital sewage (USA) (Weingarten et al., 2018), respectively, indicating a wide range of environments the species inhabits. Annotation of genes in the genome supports that AF18 possesses many common features of Enterobacteriaceae family, such as the flagella that confer mobility to the bacterium, many genetic mobile elements that facilitate transfer of stress-adaptive genes, especially those of ARGs. In fact, strains of the species have been found an important source of extended-spectrum β -lactamase-encoding genes and even carbapenem-resistant genes. For example, Enterobacteriaceae bacterium ENNIH2 was KPC-2 positive (Weingarten et al., 2018), *Metakosakonia* sp. MRY16-398 carried *bla*_{IMP-6}, *bla*_{CTX-M-2}, and *aadA2* gene (Sekizuka et al., 2018), and the [*Kluyvera*] *intestini* str. GT-16 contains many ARGs that even resistant to polymyxin (Tetz et al., 2017). A little more distant relative of AF18—*Pyrobacter ursingii* (previously named *Kluyvera intermedia*) was found to be KPC positive and carbapenem-resistant (Sheppard et al., 2016). As adapted to both wild environment and host habitats and mobility to transfer in-between, this species is able to acquire various ARGs from wild environments and when co-inhabit with their Enterobacteriaceae relatives in gut, such as *E. coli* and *K. pneumonia*, is able to share these ARGs through various genetic mobile elements or even in a more efficient manner of conjugating resistant plasmids. Thus, the species of AF18 may prove important in spreading ARGs and function as a mediator in the eco-evolutionary feedback loops of AMR. In this regard, the novel species and many other emerging opportunistic pathogens of Enterobacteriaceae family, such as *Kluyvera* spp. and *Enterobacter* spp., deserve more attention in clinical practice and the field of antibiotic resistance control.

In our study, the resistant AF18 does not have to transfer its resistance gene to the co-infected sensitive *K. pneumonia* to confer protection. Being antibiotic-resistant by itself, AF18 just upregulated the production of antibiotic-hydrolase to generate a niche of low antibiotic concentration for the sensitive *K. pneumonia* to hide. Such co-operations between different bacteria have been deeply investigated in experiments, and relevant theories or mathematic models have been fully developed which illustrated the important role of growth regulation in maximizing the benefit and population of the entire consortium (Wang et al., 2014; Scherlach and Hertweck, 2018; Li et al., 2019). Our study provides an empirical evidence for these hypothesis and highlights the importance of mutualistic relationships between co-infected microbes in clinical settings. Transcriptome analysis further identified genes involved in growth regulation and

pointed to two novel sRNAs that might be the key regulators of the process. As antibiotics are not always successful especially in treating opportunistic pathogens, the sRNAs that promote the growth of host strains as we had identified may serve as targets of bacteriostatic agents and deserve further investigations.

In conclusion, opportunistic pathogenic strain Enterobacteriaceae bacterium AF18 is in a novel species with little documentation. In-depth genomic analysis indicates the ability of this species in transferring between wild and host habitats and activity in transferring antibiotic resistant genes, which potentiates it to be an important disseminator of antibiotic resistance to clinical pathogens. When co-infected with typical pathogens, the resistant opportunistic strain is able to provide temporary protection for the whole consortium and causes confusions in the etiological diagnosis and antibiotic treatment, although the strain by itself is not a pernicious pathogen for immunocompetent patients. Taken together, Enterobacteriaceae bacterium AF18 and other newly emerging opportunistic pathogens complicate the situation of antibiotic resistance control in clinical practice and deserve in-depth investigation including methods for critical surveillance and controls on them.

Declarations

Data Availability

The complete, annotated genomic sequence of AF18 was deposited in a public database GenBank (accession numbers: chromosome, CP25982; pAF18_1, CP25983; pAF18_2, CP25984). The clean reads sequences for RNA-Seq could be downloaded from <https://bigd.big.ac.cn/gsa/s/hmrXuBu6>.

Ethics Statement

The study protocol was approved by the ethics committee of Peking university people's hospital (Approval No. 2015PHB037-01, 17/01/2015). Written consent was acquired from the patient.

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Author Contributions

Zhancheng Gao and Yu Kang designed and conducted the study. Yusheng Chen collected the samples and provided the clinical information. Zhao Zhang, Daixi Li, Yao Zhai, Yatao Guo, Lili Zhao and Yali

Zheng performed the experiments. Zhanwei Wang and Jianrong Su identified the isolate. Zhao Zhang, Xing Shi and Yukun He performed the bioinformatics analyses. Zhao Zhang, Yu Kang and Xing Shi prepared the manuscript. Zhancheng Gao revised the manuscript, and all authors read the manuscript and approved the submission.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1 The circular map of AF18 chromosome and plasmids. From the outside to the center: Genes on the forward strand, genes on the reverse strand, the annotation genes in COG database, the annotation genes in GO database, the annotation genes in KEGG database, ncRNA, GC content, GC skew.

Figure S2 The Sequences and the secondary structures of sRNAs

Table S1 The results of biochemical testing of AF18 isolate by VITEK II2

Table S2 The genome annotation results of AF18

Table S3 The summary statistics of genomic features of AF18

Figures

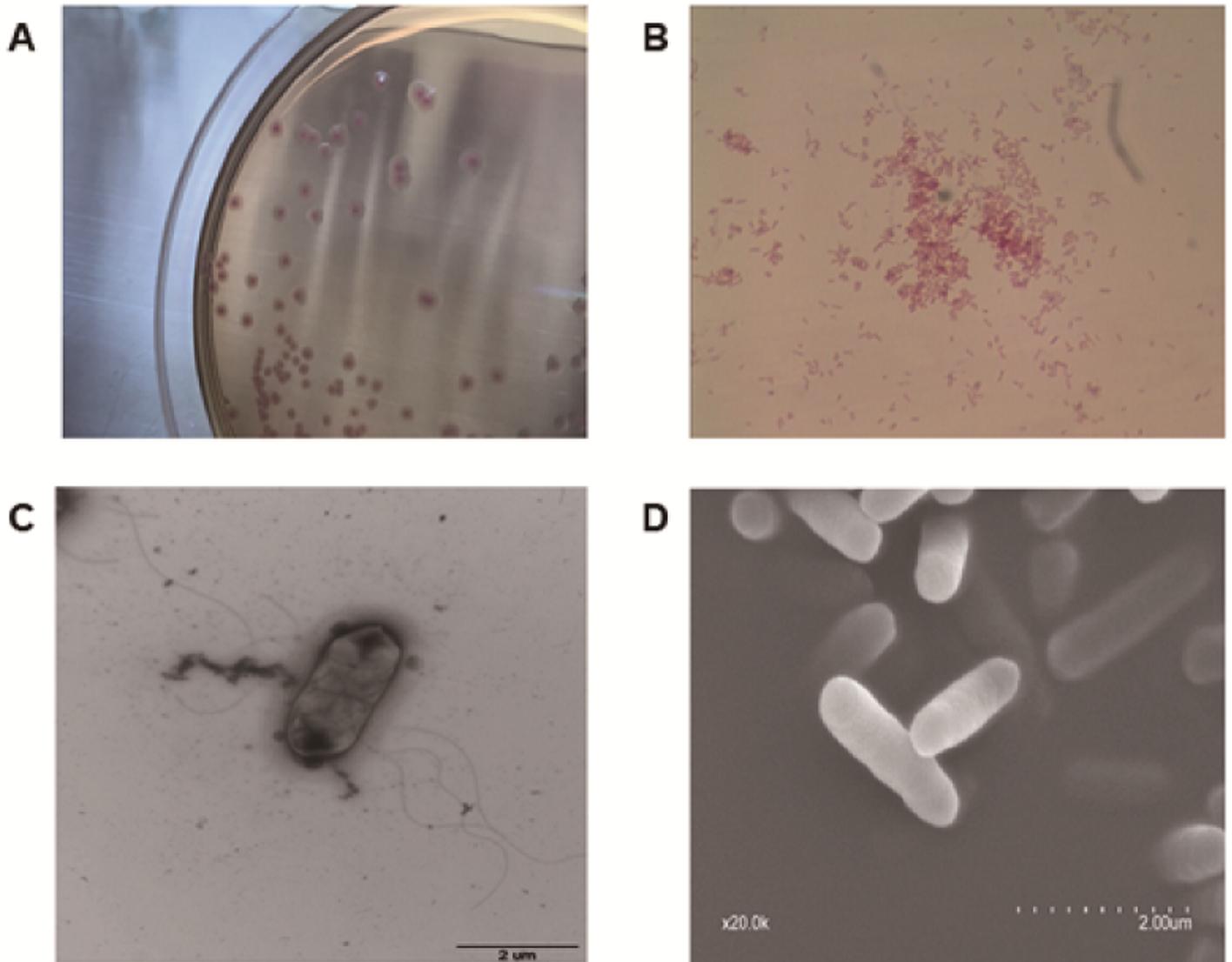


Figure 1

The morphological characters of AF18. (A) The morphology of AF18 colonies on MacConkey agar plate. (B) Gram staining of AF18 cells. (C) Flagella of AF18 photographed by transmission electron microscopy. (D) Cells of AF18 under scanning electron microscopy.

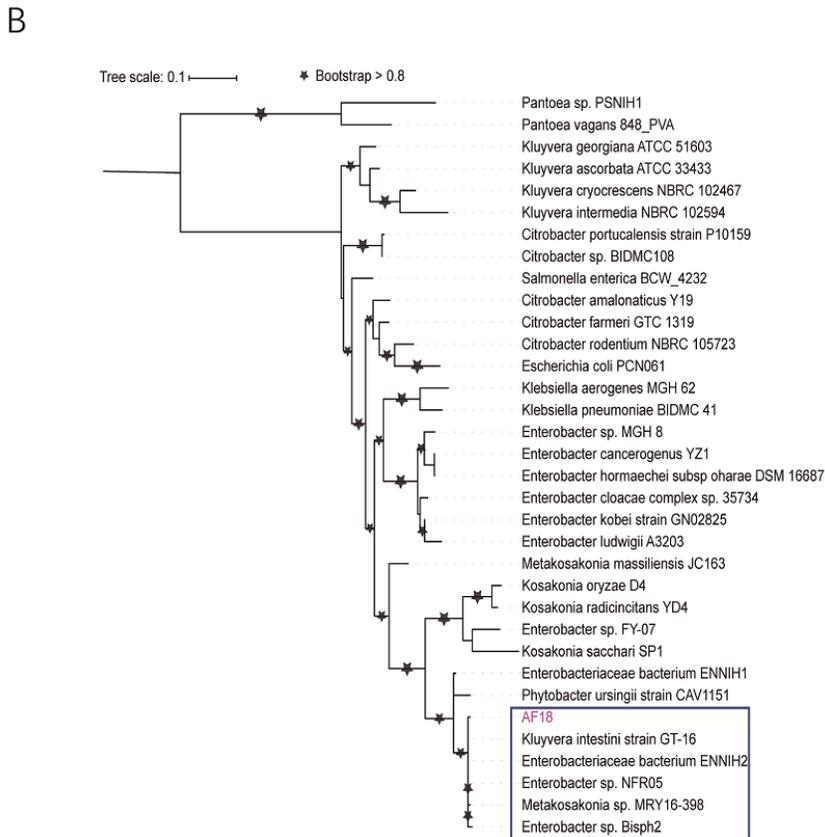
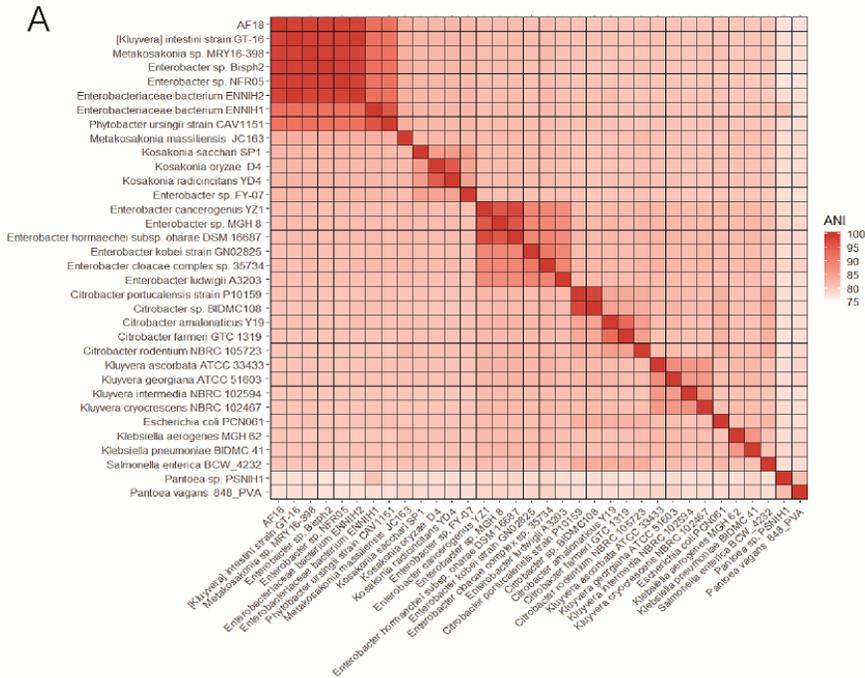


Figure 2

Phylogenetic relationship of 34 strains related to AF18. A. The heatmap of ANI matrix. Color bar represents the value of ANI. The species in blue box are the closest relatives of AF18 with ANI>98.5%. B. The maximum likelihood phylogenetic tree constructed based on the core genome SNPs. The species in blue box are the closest relatives of AF18 in the phylogenetic tree which were the same as those in the box of ANI heatmap. ANI, average nucleotide identity.

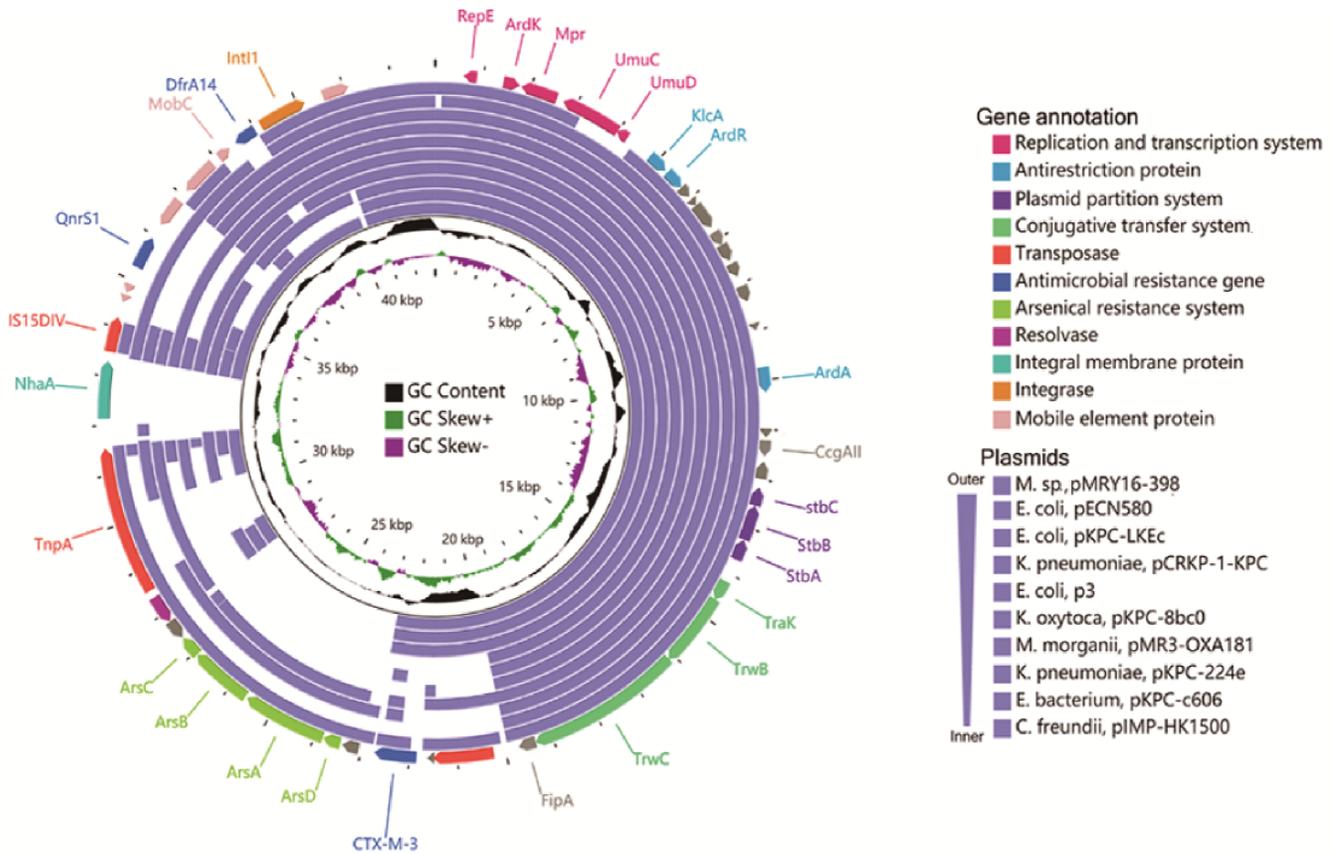


Figure 3

The circular map of pAF18_2 and comparison to similar plasmids. The outmost slot represents the predicted genes of pAF18_2, whose functions are shown in different color arrows. From outward, slot 2-11 indicate aligned fragments from similar plasmids of IncN. Slot 12, GC content; slot 13, GC skew.

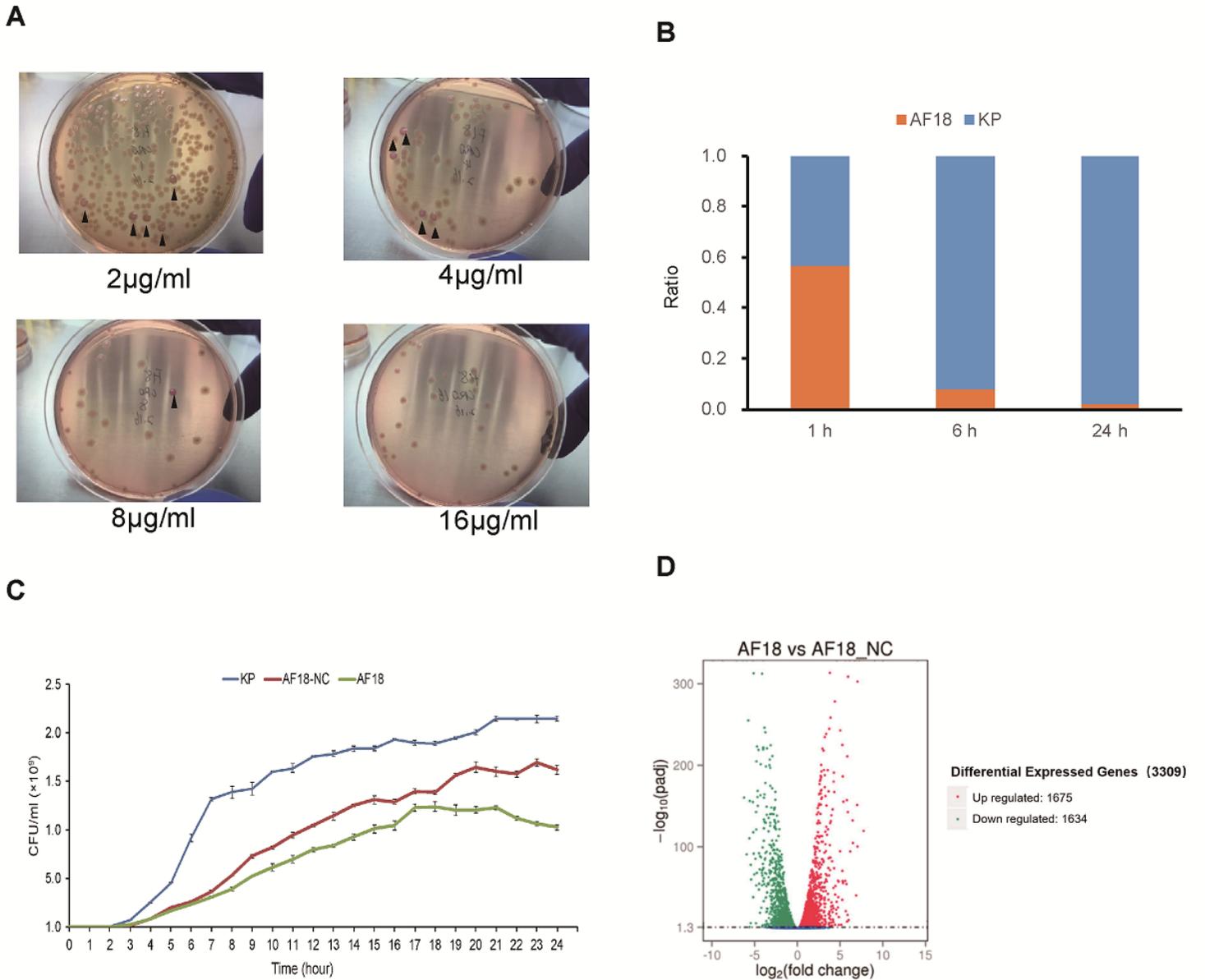


Figure 4

The properties and regulation of the growth rate of AF18. (A) Over-night co-culture of AF18 and the co-infected *K. pneumoniae* strain in LB medium was spread on MacConkey agar plates supplemented with ceftriaxone at a concentration of 2-16 µg/mL. (▲) stands for *K. pneumoniae* colonies. (B) Proportion of AF18 in the co-culture with the co-infected *K. pneumoniae* strain in LB medium without antibiotic pressure. (C) The growth curves of AF18, AF18-NC and the *K. pneumoniae* strain. (D) Up- and down-regulated genes in AF18 when compared to the transcriptome of AF18-NC.

Supplementary Files

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