The evolutionary puzzle of *Escherichia coli* ST131

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Highlights

- The pathogenic clone *Escherichia coli* Sequence Type 131 is globally disseminated.
- Reasons for its rise in provenance are not well understood experimentally.
- We report a detailed account of its biology in an evolutionary context.

Abstract

The abrupt expansion of *Escherichia coli* sequence type (ST) 131 is unmatched among Gram negative bacteria. In many ways, ST131 can be considered a real-world model for the complexities involved in the evolution of a multidrug resistant pathogen. While much progress has been made on our insights into the organism's population structure, pathogenicity and drug resistance profile, significant gaps in our knowledge remain. Whole genome studies have shed light on key mutations and genes that have been selected against the background of antibiotics, but in most cases such events are inferred and not supported by experimental data. Notable examples include the unknown fitness contribution made by specific plasmids, genomic islands and compensatory mutations. Furthermore, questions remain like why this organism in particular achieved such considerable success in such a short time span, compared to other more pathogenic and resistant clones. Herein, we document what is known regarding the genetics of this organism since its first description in 2008, but also highlight where work remains to be done for a truly comprehensive understanding of the biology of ST131, in order to account for its dramatic rise to prominence.

Keywords: *E. coli* ST131, evolution, antimicrobial resistant organisms

1. Introduction

E. coli ST131 is clinically classified as an extra-intestinal pathogenic *E. coli* (ExPEC) as it predominantly resides in the digestive tract of mammals but mediates its pathogenicity elsewhere, most commonly in urinary tracts, which can frequently progress to blood stream infections (Manges et al., 2019). It was first identified in 2008 (Coque et al., 2008; Lau et al., 2008; Nicolas-Chanoine et al., 2008), belongs to phylogroup B2 and are serotype O16:H5 or O25b:H4. Lineages of *E. coli* are categorized based on multi-locus sequence-typing (MLST), where internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) are analysed, and the alleles at each loci determine the sequence type (Nicolas-Chanoine et al., 2014). Similarity based on these biochemical

and molecular characteristics implies that isolates are clones, which share a common ancestor (Peirano and Pitout, 2019).

Within three years of its initial discovery, ST131's global distribution became apparent (Nicolas-Chanoine et al., 2014). ST131 is most frequently isolated among patients with urinary tract infections, and has been reported to account for as high as 70% of total ESBL-producing isolates, 78% of total fluoroquinolone resistant isolates and 38% of total *E. coli* isolates from clinical studies in India, the US and New Zealand respectively (Colpan et al., 2013; Hussain et al., 2012; Williamson et al., 2013). It is most commonly associated with community-onset health-care associated infections, where high levels of antibiotics pertain. Resistant ST131 carriage is particularly prevalent in long-term care facilities, with rates as high as 55%, 36% and 25% in facilities in Ireland, the UK and the US respectively (Ludden et al., 2019). As awareness of the organism increased, so too did the range of sources from which it was isolated, including animals such as companion, food-production, wildlife, avian, primate, amphibious sources, but also environmental sources such as the soil (Said et al., 2015), beach water (Vignaroli et al., 2013) and wastewater (Finn et al., 2019; Jamborova et al., 2018).

Traditionally, when organisms display such a pandemic level of distribution, it can be attributed to factors such as high virulence in the case of *Yersinia*, multi-drug resistance in the case of methicillin resistant *Staphylococcus aureus* (MRSA), or high transmission rates in the case of *Gonorrhoea*. However, ST131's virulence profile is modest based on experimental data in mice (Johnson et al., 2012). Its resistance profile is impressive, but less extensive than that of other strains of *E. coli*. Finally, its transmission rates can be kept under control by relatively benign sanitary practices among hospitalized patients (Torres et al., 2018). Evidence from epidemiological studies suggest that no single one of these parameters alone accounts for the remarkable global expansion associated with ST131, but rather a combination of factors have primed this organism for global success.

2. Population structure of ST131

Prior to whole genome sequencing, populations were classified based on phylogroups from pulse field analysis. With the onset of affordable whole genome sequencing and innovations such as long read sequencing, whole genome phylogenetic analysis of ST131 has been extensively studied. Isolates are typically collected from a wide geographical range or isolation source to identify clustering based on sequence identity. Phylogenetic analyses are performed on the core ST131 pangenome, or portion of genomes within a collection that are present among all strains. In most cases, this excludes accessory (portion of the genomes that are common, but not present in all strains) and unique (regions that are only in one strain) parts of genomes (Petty et al., 2014). While isolates cluster according to shared traits, notably antimicrobial resistance or virulence genes, analysis has proven difficult as the evolution of ST131 is heavily influenced by many factors which complicate phylogenetic analysis, in particular horizonatal gene transfer (HGT). Despite this, methodologies have been broadly similar between different studies, with many reaching comparable conclusions from different sample sources and sizes (Table 1). More recent phylogenetics have attempted to put dates on the adaptive evolution of the clades and broadly, a consensus is building.

The first report to examine ST131 phylogenetically was by Petty et al. who, with 95 different ST131 isolates, demonstrated that the population structure is broadly devided into three clades, designated according to their *fimH* allele (Petty et al., 2014). The *fim* operon encodes appendages known as fimbria, a virulence factor which bacteria use to attach to host tissue. The *fimH* gene in particular is resposible for the recognision and attachment to the manose receptors on host tissue (Krogfelt et al., 1990). The gene is highly variable due to its location on a region of high recombination and different alleles correlate stongly with the population subtypes of ST131.

Table 1: Details of the population structure of ST131. Fq fluoroquinolone, ESBL extended specturm β-lactamase

Clade	Serotype	Predominant *fimH* allele	Also known as	Estimated timeline of origin or divergence (95% HPD interval) (Ludden et al., 2019)	Frequently associated IncF plasmid (Johnson et al., 2016)	Resistance profile
A	O16:H5	41	-	-	F29:B10	None
B	O25b:H4	22	-	1901 (1842 – 1948)	F29:B10	None
C0	O25b:H4	30	H30	1985 (1980 – 1989)	F2:A1:B-	None
C1	O25b:H4	30	H30-R	1994 (1991 – 1996)	F1:A2:B20	Fq, ESBL (CTX-M-14 or -27)
C2	O25b:H4	30	H30-Rx	1994 (1991 – 1996)	F2:A1:B- (integrated)	Fq, ESBL (CTX-M-15)

*HPD – highest posterior density

2.1 Clade A

Clade A ST131 have the *fimH*$_{41}$ allelle. They may have originated in South East Asia (Stoesser et al., 2016) in the late 1880's, and are the most closely related *E. coli* type to clades B and C. Despite this, clade A are distinct from clades B and C based on and maximum likelihood phylogenetic analysis of the ST131 core genome. They are generally sensitive to antibiotics. A consequence of this is that their true numbers and distribution may be underappreciated, as the majority of large scale screening studies in the past have used antibiotic resistance as a selectable marker for the detection of other more resistant clades of ST131. Recent evidence suggests clade A strains appear to occupy distinct ecological niches (Finn et al., 2019; McNally et al., 2019), likely due to differences in the accessory genome when compared to clades B and C.

2.2 Clade B

Clade B strains predominantly have the *fimH*$_{22}$ allele, although there are instances of *fimH*$_{35}$ in this clade (Peirano and Pitout, 2019). Clade B are thought to have emerged from clade A in the early 1900's (Ludden et al., 2019), originating from North America (Stoesser et al., 2016). Clade B are generally antibiotic sensitive and as in the case for clade A, their true distribution based on large scale screening studies may also be underrepresented. From Zakour et al, intermediate sub-clades from B0 to B5 were identified within the B clade (Zakour et al., 2016). Despite having a conserved virulence gene profile, B intermediates vary in their single nucleotide polymorphism, mobile genetic element and recombination profile, and display a stepwise acquisition, with instances of *fimH*$_{30}$ in sub-clade B0, likely leading to the evolution of clade C (Decano and Downing, 2019).

2.3 Clade C

2.3.1 Clade C0

Clade C are *fimH*$_{30}$ and emerged from clade B in the mid 1980's (Ludden et al., 2019). The origin of clade C is thought to be from the US or Canada (Zakour et al., 2016). Within seven years, this clade diverged into subclades C0, C1 and C2 (Johnson et al., 2016). Zakour et al. identified two key events thought to be responsible for this diversification. The first was a recombination-based mutation in

parC1, followed by the integration of genomic islands LeuX, PheV and the prophage Phi1 around the same time the *fimH*$_{30}$ allele emerged, and are thought to play a key role in establishing C clades clones for later success (Pitout and DeVinney, 2017). The major difference between C0 and daughter clades C1 and C2 is in their susceptibilities to fluoroquinolone antibiotics. The original C0 arose before the clinical use of fluoroquinolones and so is sensitive to this class of antibiotics that were used extensively in human medicine for the treatment of UTIs and also in veterinary medicine (Zakour et al., 2016).

2.3.2 Clade C1

Clade C1 and C2 are generally considered the globally successful strains based on their widespread geographical distribution. Both clade C1 and C2 strains are resistant to fluoroquinolones as a result of two chromosomal mutations. The first is a mutation in *gyrA*, which encodes DNA gyrase subunit A. When the protein is in its tetrameric form, it negatively supercoils doublestranded DNA and is the target of fluroquinolone antibiotics. There are five different alleles of mutated *gyrA* that predominantly arise in ST131. The allele selected for in clade C1 and C2 is *gyrA*-1AB, where the 87th amino acid of the polar asparagine amino acid is replaced by a negatively charged aspartic acid. Strains with identical *gyrA* mutations have been observed to have varied fluoroquinolone minimum inhibitory concentrations, indicating that further mutations outside *gyrA* contribute to resistance also (Weigel et al., 1998).

A second gene responsible for fluoroquinolone resistance in ST131 is *parC*, which encodes DNA topoisomerase IV subunit A. There are six frequently observed *parC* alleles in ST131, and the allele selected for in clade C is *parC-1aAB*, where the serine (polar uncharged) residue at position 80 is changed to an isoleucine (hydrophobic), and the 84th residue a glutamic acid (negative) is changed to valine (hydrophobic), mediating resistance to fluoroquinolones (Stoesser et al., 2016). Both the *gyrA* and *parC* mutations were likely introduced into clade C via recombination (Petty et al., 2014) and are vertically transmitted. There is debate as to whether resistance emerged before or after widespread

use of fluoroquinolones in 1986 (Zakour et al., 2016). Nevertheless, fluoroquinolone resistance is a major determining factor of clade C ST131.

Broadly, C1 and C2 clades differ from each other based on genes that have been aquired from different mobile genetic elements (Johnson et al., 2016). Key features present in clade C1 and not in C2 include plasmid stability genes *sopAB* and *psiAB*, and island 1 and a 10-kb region containing the colicin Colla immunity-encoding gene *cjrABC*, and the enterotoxin-encoding *senB* gene (Johnson et al., 2016).

2.3.3 Clade C2

While both clade C1 and C2 are fluoroquinolone resistant, C2 have a very strong association with extended spectrum β-lactamse (ESBL) production. This is thought to have arisen when a resident plasmid already within the C2 sublineage, acquired an insertion element-mediated gene cassette containing ESBL-producing genes. This was a pivotal moment in the evolution of clade C2, as it marks the beginning of the association between ST131 and ESBLs, which after this linkage and co-selection, increased the prevalence of both ST131 and ESBLs worldwide. This multidrug resistant gene later integrated into the chromosome of some clade C2 strains (Petty et al., 2014). Subsequently, clade C1 became associated with an alternative ESBL (see 6.2 Additional resistance genes).

3. ST131 epidemiology based on phylogenetic studies

Despite making great progress in outlining the population structure of ST131, phylogenetic studies have not been as successful in identifying an epidemiological origin for the strain. Increased resoultion of the ST131 population structure was possible by considering both the core and accessory genome (Decano and Downing, 2019; McNally et al., 2016a). These confirm that ST131 is a host generalist capable of frequent interspecies movement. However, lack of clade diversity and under-represented samples collected from sources other than human isolates, mean that no definitive origin or host reservoir was uncovered. The lack of temporal or geographical clustering of samples suggest a complex evolutionary history. Overall, ST131 has emerged through a combination

of clonal expansion from fluoroquinolone resistance and multiple horizontal gene transfer events containing ESBL alleles (Johnson et al., 2016).

4. Virulence

There is conflicting evidence as to the virulence potential of ST131 (Mathers et al., 2015). Virulence genes are typically associated with the accessory genome due to the movement of mobile genetic elements such as plasmids or genomic islands and thus, contribute to inter- and intra- species variation (Dobrindt, 2005). Few virulence genes appear to be uniformly present in ST131 (Petty et al., 2014), and those that are present are mostly associated with other ExPEC. Of the 40 ExPEC-associated virulence genes, only 13 were detected in ST131 (Horcajada et al., 2005). Blanco et al classified 195 ST131 samples into four virotypes (A to D) based on the presence or absence of four genes (Blanco et al., 2013); i) Afa FM955459 an afa/dr adhesin, ii) *iroN* a catecholate siderophore receptor, iii) *ibeA* an invasion of brain endothelium and iv) *sat* a secreted autotransporter toxin. When this is broken down by clade, clade C were all virotype A, B or C, while clade B were all virotype D. Interestingly, clade B strains had the highest virulence score based on this classification. Conversely, others have found that ST131 may be more proficient at biofilm production than other *E.coli* (Clermont et al., 2008; Kudinha et al., 2013), which may be an important factor in its ability to establish and maintain intestinal colonization. However, experimental animal models do not support ST131 as being more virulant than other *E. coli* (Banerjee and Johnson, 2014). When ST131 and non-ST131 *E. coli* were compared in a mouse subcutaneous sepsis model, the presence of ST131 was not found to correlate with illness severity (Johnson et al., 2012).

Many ExPEC virulence factors serve dual roles for bacteria residing in the colon, in that they may confer advantages for adaptation to this environment (eg *fimH* attachment), rather than for infection or toxin production in the case of LEE pathogenicity islands or Shiga toxin production (Mokady et al., 2005). Furthermore, virulence is more intricate than simply the presence or absence of virulence genes, expression levels should also be considered (Pitout, 2012). Overall, it seems the virulence capability of ST131 is focused more on persistance in the host, rather than disease.

5. Mobile genetic elements

5.1 Plasmids

Plasmids have had a transformative impact on the evolution of ST131. Plasmids are divided into incompatibility (Inc) groups based on their inability to co-exist in the same cell. They can be further sub-classified based on their intrinsic replication regions or replicons. Plasmids that seem to be associated with ST131 are the IncF group, named after the production of F type pili, of which there are six groups (IncFII to VII) (Carattoli, 2009). General features of IncF plasmids include frequently containing multiple origins or replicons, they usually are greater than 100 kb in length, have low copy number, typically contain *pemI/pemK* addiction systems, often carry virulence or antimicrobial resistance genes and are mostly restircted to the *Enterobacteraceae* (Villa et al., 2010).

Unravelling the association between ST131 and IncF plasmids using sequence data consisting of highly plastic elements has been challenging, but with single molecule real time sequencing, the complex evolutionary relationship has become more clear (Johnson et al., 2016) (Figure 1). While clade A and clade B ST131 are not always associated with plasmids, there is an association with F29:B10 plasmids. F29:B10 may have been lost before the emergence of clade C0. In contrast to clade A and B, there is a very strong association between clade C and the IncF plasmids. C0 gained F2:A1:B-. This plasmid was then lost in the trajectory resulting in the evolution of C1, but replaced with F1:A2:B20 (Johnson et al., 2016). For the evolution of C2 however, F2:A1:B- remained, and at some point acquired the gene cassette containing $bla_{\text{CTX-M-15}}$ through an IS26 insertion elements (Stoesser et al., 2016). It may also have contained other antimicrobial resistant genes *catB4*, $bla_{\text{OXA-1}}$ and *aac(6')Ib-cr*, reducing susceptibility to chloramphenicol, oxacillin and aminoglyocsides respectively. In some cases, the IS26 stabily integrated into the genome and there is evidence of extensive rearrangements since (Johnson et al., 2016). Col-like plasmids are a subset of IncF plasmids that are so named as they produce colicin V, which enhance the ability of bacterial strains to survive and proliferate in the tissue and fluids of infected animals, particularly birds (Williams and Warner, 1980). Recently, a strong association of ColV plasmid with clade B mediated a form of avian

specialization within this clade (Liu et al., 2018). There are also a number of non-IncF plasmids identified in ST131 (Stoesser et al., 2016). Other plasmids that are known to occur include IncA/C, L/M and N and are most likely transferred from different species (Mathers et al., 2015). IncH has been associated with clade B and IncI with clade C1.

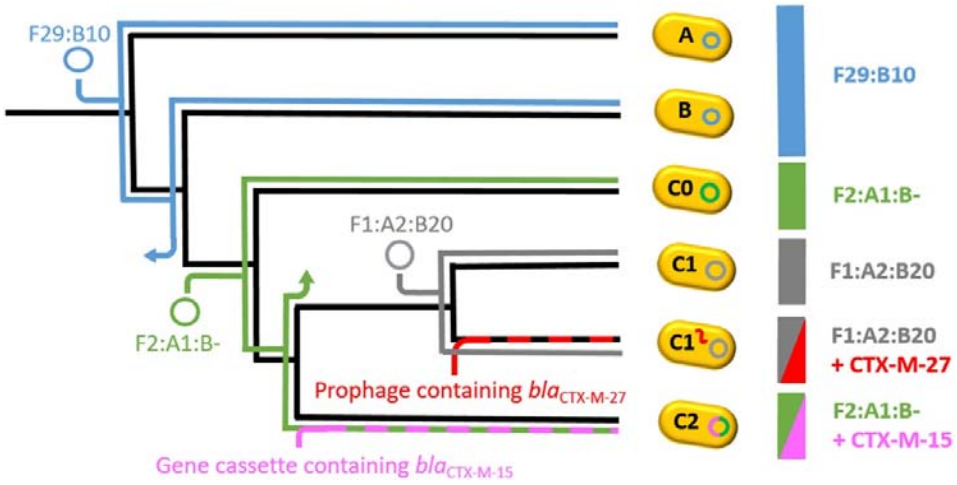

Fig. 1. Dendrogram representing the acquisition of critical IncF plasmids and ESBL-containing mobile genetic elements in different ST131 clades. Black solid lines indicate descent of ST131, coloured lines represent line of descent of plasmids or ESBL-containing mobile genetic elements. Dashed lines represent integration into either the host chromosome or plasmid. Coloured boxes represent plasmids and CTX-M genes most commonly associated with each clade. Figure adapted from (Johnson et al., 2016).

In bacteria, plasmid carriage often comes with a tradeoff in fitness via declined growth rates (da Silva and Bailey, 1986; Lenski, 2007). Generally, this is attributed to plasmids competing with the host cell for resources involved in translation (Bragg and Wagner, 2009; Shachrai et al., 2010), cytotoxic effects due the presence of the plasmid (Baltrus, 2013), or regulatory interations between plasmid and bacteria (Baños et al., 2009; Doyle et al., 2007). However, recent studies are challenging this dogma, instead finding that certain plasmids do not decrease fitness in ST131 (Johnson et al., 2016; Schaufler et al., 2016; Shin and Ko, 2015). Shin and Ko transformed clinical strains of ST131 with plasmids containing either $bla_{CTX-M-14}$ or $bla_{CTX-M-15}$ and were compared to ESBL-sensitive parent strains. Authors observed a decrease in virulence, in particular the capability of the strains to form

biofilms (Shin and Ko, 2015). However, there were no differences in either growth rate or relative fitness when competed in minimal media. This finding is broadly supported by Schaufler et al, although conversley, they reported enhanced effects on virulence. *In vitro* fitness differences of plasmid carriage was also investigated by Johnson 2016, particularly among the three clade C striains (Johnson et al., 2016). When the plasmid of a C2 strain was introduced into strains from C0 and C1 and competed against K12 in rich media, fitness costs were found in C0 but no significant costs in fitness were observed in the C1 strain, which could implicate the existance of mutations in the genetic background that mitigate the fitness cost of plasmid carriage.

Conflicting results between studies could be due to lack of consistancy with competition conditions, different genetic backgrounds and/or differences between the gene content of various plasmids used. These results highlight the complex epistatic network of interactions that exist between plasmid genes, host genes, addiction systems etc, each contributing to the overall fitness of the strain, but to an unknown degree. Nevertheless, plasmids seem to coexist with ST131 in a balanced mutualism.

5.1.1 Compensatory mutations

The lack of fitness decline in ST131 harbouring large plasmids may not be a property of the plasmid, but an adaptive feature of the host. Compensatory mutations are those that offset the negative effect of previously occuring mutations. They can also promote stable plasmid carriage that would otherwise have negative effects on the fitness of the host strain (Bouma and Lenski, 1988; Dahlberg and Chao, 2003; Millan et al., 2014). Typically, compensatory mutations affect genes encoding proteins involved in cellular functions affected by either the original mutation (Durão et al., 2018) or the accessory genes contained on the plasmids (Harrison et al., 2015). A common feature of compensatory mutations is the more costly the initial mutation, the more rapid the response of the compensatory mutation, and plasmid stability can be enhanced in this way.

In *Pseudomonas fluorescens*, the pQBR103 plasmid confirs resistance to mercury due to the *mer* operon, but at 425 kb, it also imposes a significant translational demand on the host. In experimentally evolved lines of *P. fluorescens*, deletions of a sensory kinase *gacA*, or it's response regulator for secondary metabolites *gacS*, aleviated this metabolic burden leading to stable coexistance of the initally deleterious plasmid (Harrison et al., 2015). Comparatively little is known about compensatory mutations in ST131. When the accessory genome of ST131 is investigated more closely, clade A and B accessory genomes are more similar to each other than to C (McNally et al., 2016a). Authors postulate that the conserved accessory genome of clade C could be the location of compensatory mutations, necessary for the carriage of resistance plasmids and phages associated with this clade, which controls the expression of nearby genes within the core genome. Alternatively, the mutation in DNA gyrase found in clade C could also be a compensatory mutation, in addition to mediating fluoroquinolone resistance. DNA gyrase is a host-encoded protein, required for efficient replication for IncF plasmids (Toukdarian, 2004; Villa et al., 2010), potentially the mutated *gyrA* allele present in clade C2 strains could allow for stronger association between the plasmid and host.

5.2 Genomic islands and prophages

Genomic islands are any part of a genome for which there is evidence of horizontal origin. Prophages are a subset of genomic islands, where bacteriophage genomes have inserted into a bacterial cell, as either an extrachromosomal plasmid or integrated directly into the host chromosome. They are identified bioinformatically by a variety of hallmark features including high AT content and/or the presence of certain phage genetic signaturtes like integrase genes or direct repeats (Bertelli et al., 2017). Their dissemination throughout bacterial species is mostly through transduction or conjugation (Hochhut et al., 2006). Genomic islands play a key role in bacterial evolution due to their large size and gene content and accordingly, have a dramatic influence on the phenotype of the host. Among atypical enteropathogenic *E. coli* (aEPEC), the presence of single genomic islands such

as the locus of enterocyte effacement (LEE) pathogenicity island was identified as the major source of variantion between outbreak and non-outbreak strains (Ingle et al., 2016).

Owing to the many large scale phylogenetic studies of ST131, genomic island integrations have been well documented in the evolutionary trajectory of ST131. The Flag-2 island entered into clade B strains around 1950, and contains genes for a type three secretion systems and flagelar assembley confering motility (Ren et al., 2005). Specifically for clade C, three genomic islands have been pivotal in diversification from clade B to C, known as Phi1, PheV and LeuX (Zakour et al., 2016). This event is estimated to have occurred around 1980, at the same time as the origin of the *fimH*$_{30}$ allele and just before the the *gyrA* and *parC* mutations. The prophage Phi1 is 37 kb in length, and contains over 50 genes, mostly phage-associated. PheV is 74.5 kb and contains over 70 genes, with a high proportion of virulence, pathogen-associated and resistance genes including *ag43* involved in biofilm formation, *papI* encoding pilli and *bla*$_{\text{CMY-23}}$ a β-lactamase, respectively. LeuX is 93 kb and contains over 90 genes. It is located in a highly variable region of the ST131 genome close to the *fimH* gene.

In addition to their role providing a source of novel traits, genomic islands are responsible for recombinant regions in the chromosome. According to BEAST, ST131 has a mutation rate of one mutation per genome per year (Stoesser et al., 2016), with these regions accounting for the majority of the SNP-based diversity among clades. While much of the attention has focused on pathogenicity and virulence characteristics, more recently investigation has shifted to the role these islands have on fitness (Diard et al., 2010). However, specifically for ST131, little is known about the role these islands have on the overall fitness, despite the important role these acquisition events had in shaping the its population structure and driving SNP-based mutations.

6. Antimicrobial resistance

Several studies have demonstrated that the presence of antibiotics in the environment offers strong selection pressure for the emergence of resistant forms, which can go on to become highly successful strains (Cunha et al., 2014; He et al., 2013; Holden et al., 2013; Redgrave et al., 2014).

Comparative genome analysis and phylogenetic reconstruction of *Streptococcus agalactiae*, a leading cause of bacterial neonatal infections in Europe and North America was performed (Cunha et al., 2014). Authors identified the evolution of tetracycline resistant clones, which replaced a previously diverse population, and gave rise to the worldwide dissemination of only a few tetracycline resistant clones in the 1950's. Mutations confering fluoroquinolone resistance in particular seem to trigger the emergence of highly successful clones that have established in a range of environments (Redgrave et al., 2014). These include *S. aureus* strain EMRSA-15 and *C. difficile* 027 FQR1 and FQR2 lineages. *S. aureus* strain EMRSA-15 was responsible for 60% of nosocomial infections in England (Holden et al., 2013). Investigation of the highly successful EMRSA-15 clone identified two mutations mediating resistance to fluoroquinolone, one in *grlA* resulting in an S80F, the other in *gyrA* consisting of a S84L and are thought to have played a role in the success of the clone, even though fluoroquinolones are rarely used to treat *S. aureus* infections. In the case of *C. difficile* O27, comparative genomic analysis between two highly successful clones found only one similarity, both had mutations in the *gyrA* gene, mediating resistance to fluoroquinolone. *C. difficile* O27 is now a leading cause of antibiotic resistant diarrhoea worldwide (He et al., 2013).

6.1 Does fluoroquinolone resistance prime a species for success?

The molecular mechanisms mediating resistance to fluoroquinolones are well understood. Fluoroquinolone antibiotics inhibit two topoisomerase enzymes; DNA gyrase, which introduces negative supercoils (Gellert et al., 1976) and topoisomerase IV, which relaxes DNA (Zechiedrich et al., 2000). Modification of either target renders the cell resistant and is likely to affect the supercoiling ability of the organism. By comparison, the biological context for the enhanced beneficial fitness effects as a result of fluoroquinolone resistance are not as well defined. From microbial evolution studies, increased supercoiling is a trait known to improve fitness of long term evolved lines of *E. coli*, even in the absence of antibiotic exposure (Crozat et al., 2005). Increased fitness is hypothesized to result from tighter control of the expression levels of many genes

simultaneously. Furthermore, these experimental evolution studies also confirmed that multiple mutations affecting DNA supercoiling can contribute additively to overall fitness of the organism.

6.2 Additional resistance genes

In addition to ST131's well documented resistance to fluoroquinolones, clades C1 and C2 are further resistant to β-lactam based antibiotics. β-lactams are antimicrobial agents that bind and inhibit bacterial enzymes involved in cell wall biosynthesis. β-lactamases are bacterial enzymes that inactivate β-lactam antibiotics, usually by hydrolysis (Peirano and Pitout, 2019). Overall, there are five main types of β-lactamases that vary based on molecular and functional properties, i) ESBLs, ii) AmpC, iii) Metallo-enzymes, iv) KPC (*Klebsiella pneumoniae* carbapenemase)-like, and v) OXA (resistant to oxacillin)-like (Table 2). By definition, ESBLs are β-lactamases with activity against oxyimino-cephalosporins and monobactams, and are inhibited by classical β-lactamase inhibitors such as clavulanic acid. While each type has been associated with ST131, the extent of their association varies greatly and the reasons for this are not well understood.

Previous to the 1990's the most common types of ESBLs were *bla*$_{SHV}$ (sulfhydryl-variable) and *bla*$_{TEM}$ (Temoneira, after the first patient from which it was isolated). In 2003, a new enzyme emerged that preferentially hydrolyses cefotaxime, encoded by *bla*$_{CTX\text{-}M\text{-}15}$ (active on cefotaxime, first isolated in Munich) and gained prominence over the 2000s (Pitout, 2012). The *bla* genes encode enzymes that hydrolyse penicillins, cephalosporins and monobactams, but cannot hydrolyse cephamycins or carbapenems and are inhibited by β-lactamase inhibitors like clavulanic acid. There are over 172 CTX-Ms identified and described (Ramadan et al., 2019), grouped into five clusters based on their amino acid identities (Boyd et al., 2004). Different CTX-M alleles are associated with different ST131 clades. Predominantly, clade C1 is associated with CTX-M-14, while clade C2 is associated with CTX-M-15. In clade C1, another variant CTX-M-27, has recently emerged. This was initially reported in Japan in 2006 (Matsumura et al., 2015), has since been identified worldwide (Courpon-Claudinon et al., 2011; Decano and Downing, 2019; Matsumura et al., 2016; Seiffert et al., 2013). The *bla*$_{CTX\text{-}M\text{-}27}$ differs from *bla*$_{CTX\text{-}M\text{-}14}$ by one nucleotide, which results in a single amino acid substitution, D240G

and provides higher levels of resistance to ceftazidime (Bonnet et al., 2003). The original CTX-M-27 clone seems to have emerged as a distinct subclade from C1, due to the insertion of the M27PP1 prophage containing the *bla*$_{\text{CTX-M-27}}$ variant (Matsumura et al., 2016).

Screening for ESBL-producing strains is technically straightforward and relatively commonplace. As a result, surveillance of ESBL-producing ST131 has been effective and widespread, to the point where there could be an overestimation of the proportion of ESBL-producing ST131, relative to non-ESBL-producing ST131. However, it remains an open question as to why certain alleles of the ESBL family have more successful established than others.

Table 2: β-lactamase enzymes and their association with ST131

Enzyme class	Examples	Carbapenem resistant	References associated with ST131
Extended spectrum β-lactamases	CTX-M, TEM, SHV	No	(Nojoomi et al., 2017)
AmpC	CMY, MOX, DHA	No	(Matsumura et al., 2012)
Metallo-enzymes	NDM-1, IMP-1, VIM	Yes	(Peirano et al., 2011)
KPC-like	KPC-2, KPC-3	Yes	(Naas et al., 2011)
OXA-like	OXA-48, OXA-181	Yes	(Morris et al., 2012)

6.2.1 Carbapenem resistance

Carbapenemases are enzymes with activity against the carbapenems, but additionally are resilient to some β-lactamase inhibitors (Queenan and Bush, 2007). They are classified into three groups, broadly based on their spectrum of activity and inhibition properties. i) Metallo-enzymes such as NDM-1 or IMP-14 are enzymes requiring metal cofactors that hydrolyse β-lactams (except aztreonam), are not inhibited by β-lactam inhibitors but are by EDTA (Nordmann et al., 2011). ii) KPCs, like KPC-2 and -3 are mostly plasmid bound genes encoding enzymes that hydrolyse β-lactams, are only weakly inhibited by β-lactam inhibitors (Queenan and Bush, 2007), have a high prevalence in *Klebsiella* spp and to a lesser extent *Enterobacter* spp. and are globally distributed. iii) The OXA group consists of over 400 β-lactamases commonly identified in *Acinetobacter* spp. with only a subset comprising of enzymes capable of carbapenemase activity, referred to as carbapenem-hydrolyzing class D β-lactamases, with the OXA-48-like types such as OXA-48 and OXA-181 being the most common in *Enterobacterales* (Pitout et al., 2019). OXAs are not inhibited by most β-lactam inhibitors or EDTA, but are inhibited by sodium chloride. The majority of these genes are located on plasmids and are of concern because they may enter already high risk clones and proliferate, as was the case with $bla_{\text{CTX-M-15}}$ and ST131. Each type of these carbapenemases have already been identified in ST131 (Peirano et al., 2014). Interestingly, these isolates have not gained prominence on the extensive scale seen with the emergence of CTX-M in the 2000's.

The success of ST131 with an IncF plasmid containing $bla_{\text{CTX-M-15}}$ is a text-book example of co-selection, but the question remains, why this particular combination has been so successful against the variety of alternative ESBL or carbapenemase options available. It seems likely that the successful combination of ST131 with CTX-M-15 on an IncF plasmid is rooted in the stable interaction between the host and the plasmid, with its suite of permissive addiction systems and toxin antitoxin genes. The ESBL allele may be of less significance and likely arose due to genetic drift. Some ESBL-encoding genes are located on plasmids that are not stably maintained in ST131. OXA genes are typically associated with IncL/M plasmids in *Klebsiella* (Poirel et al., 2012), rather than the

better suited IncF plasmids in ST131. However, ESBL genes can be located on transposons like Tn1999, leading to concerns that the gene could be horizontally transferred from plasmids in *Klebsiella* to plasmids or the chromosome of ST131. If this were to happen, are we likely to see a similar explosive evolutionary trajectory as was seen for ST131 with $bla_{CTX\text{-}M\text{-}15}$ in the 2000's? There could also be an element of clonal interference involved in this dynamic, whereby beneficial mutations such as $bla_{CTX\text{-}M\text{-}15}$ have been so dominant, that clones containing alternative variants were outcompeted, did not emerge or were not detected at a high rate.

Strains of ST131 that are capable of carbapenemase activity are typically those that already possess CTX-M. In general, it is not known if these carbapenemase enzymes are located on the same ESBL-containing plasmid or different plasmids entirely. In the latter case, acquiring additional resistance plasmids leads to increasing plasmid acquisition costs, putting an upper limit on the extent of plasmid-mediated resistance that an organism can achieve (Vogwill and Maclean, 2015). Lack of additional compensatory mutations could also explain why ST131 with additional resistance plasmids have not proliferated, however continued investigation is needed to identify the range of compensatory mutations present in ST131. Furthermore, the potential of the microbiome to harbour antimicrobial resistant plasmids is immense and currently, we have very little predictive knowledge of the probability that a given plasmid or gene will be transferred by HGT into a nascent human pathogen residing in the gut.

7. Evolutionary dynamics among resistant strains

Classification as a high risk clone requires six criteria to be met (Mathers et al., 2015). The clone must; i) have a global distribution, ii) be associated with multiple antimicrobial resistance determinants, iii) have the ability to colonize and persist in a host for over six months, iv) be capable of effective transmission between hosts, v) have ehanced pathogenicity and fitness and vi) have the ability to cause severe or recurrent infections. In many ways, the success of ST131 is surprising in that when this clone is compared to others, it is neither the most virulent, nor the most resistant. In particular, there are four *E. coli* clones that appear to have similar virulence profiles but enhanced

Table 3: Comparison of multidrug resistant strains compared to ST131. Predominant alleles are in parentheses.

	Resistance Determinants					
		ESBL				
Strain	Fq	CTX-M	NDM	OXA	mcr	References
ST131 Clade C2	Yes	Yes (15)	-	-	-	(Zakour et al., 2016)
ST405	Yes	Yes* (15)	-	-	-	(Matsumura et al., 2013)
ST648	Yes	Yes (15)	-	-	Yes (1)	(Sato, Toyotaka, Yuuki Suzuki, Shiraishi Tsukasa et al., 2016)
ST410	Yes	Yes (15)	Yes (5)	Yes (181)	Yes (1)	(Roer et al., 2018)
ST345	Yes	Yes (15)	-	-	Yes (1)	(Casella et al., 2017)

*Higher proportion of CTX-M when compared to ST131

resistance profiles, but do not display the impressive global distribution, characteristic of ST131. These strains are ST405, ST648, ST410 and ST345, and their resistance determinants are compared to that of ST131 in Table 3.

7.1 ST405

ST405 has been reported in Japan, Egypt, Italy, Saudi Arabia and Columbia (Alghoribi et al., 2015; D'Andrea et al., 2011; Fam et al., 2010; Ruiz et al., 2011). In a multicentre surveillance study in Japan, a higher proportion of strains were positive for *bla* genes, particularly CTX-M-15 and -14 in ST405 than in ST131 (Matsumura et al., 2013). Additionally, while ST405 and ST131 seem to share similar resistance profiles on the basis of presence or absence of genes, authors found a higher rate of multidrug resistance (defined as resistance to three or more antibiotics) in ST405 than ST131, with 56% of ST405 strains compared to 22% of ST131 strains meeting this criteria. ST405 isolates also displayed a higher proportion of resistance to fluoroquinolones than ST131, as well as a wider range of mutations mediating this resistance. Overall, ST405 has the potential to expand as another public health concern, but this has not been the case.

7.2 ST648

ST648 has been identified in Brazil, Japan and China (Fernandes et al., 2018; Sato, Toyotaka, Yuuki Suzuki, Shiraishi Tsukasa et al., 2016; Zhang et al., 2016). ST648 carry ESBL, but unlike ST131, they additionally contain the *mcr-1* gene, likely to be located on a plasmid. The *mcr-1* gene mediates resistance to polymixins like colistin, a group of antibiotics currently considered the last line of defence against ESBL- and carbapenemase-producing Gram negatives (Zhang et al., 2016). In addition, fluoroquinolone resistant strains of ST648 also acquired resistance to the tetracycline derivative tigecycline via expression of efflux pump AcrAB-TolC (Sato, Toyotaka, Yuuki Suzuki, Shiraishi Tsukasa et al., 2016). When directly compared to ST131, ST648 displayed similar colonization rates from *in vivo* poultry experiments, but had superior biofilm producing capabilities (Schaufler et al., 2019). Schaufler et al. investigated the role of plasmids in ST648 by curing large ESBL-containing plasmids from strains of ST131 and ST648, then compared wild type and plasmid-cured strains under a variety of growth-based phenotypic tests (Schaufler et al., 2016). Overall, wild

type strains displayed lower curli fimbriae and cellulose production, as well as reduced swimming capacity. Flagellar biosynthesis is metabolically costly, and there may be tradeoffs, when the cell needs to economize between plasmid carriage over motility when energy resources are scarce. Authors note consistent results were difficult to extrapolate, which highlights the importance of a conserved genetic background when comparing different strains and performing these assays.

7.3 ST410

ST410 was first identified in China in 2016 (Qin et al., 2016) and has since been isolated in Denmark, UK, America, Germany, Canada and Brazil (Roer et al., 2018). Roer et al reconstructed the phylogeny of another multidrug resistant clone called ST410, by investigating whole genome sequences of 127 worldwide isolates and used BEAST to estimate timelines of evolutionary acquisitions. There are two main clades, clade A ($fimH_{53}$) and clade B ($fimH_{24}$), with B clades subdivided based on different antibiotic resistance characteristics. In 1987, similar to ST131, B2 acquired fluoroquinolone resistance based on chromosomal mutations (S83L and D87N in *gyrA*, S80I in *parC* and S458A in *parE*). Around the same time, B3 aquired $bla_{\text{CTX-M-15}}$, from the IncFII plasmid. In contrast to ST131, ST410 is resistant to two types of carbapenems as clade B4 aquired bla_{OXA181} as a result of the IncX3 plasmids and in 2003, B4 also acquired $bla_{\text{NDM-5}}$ from an IncA/C plasmid in 2014. Additionally, some ST410 strains from Brazil and Germany have been reported to have the mobile colistin resistance gene *mcr-1* (Falgenhauer et al., 2016b; Rocha et al., 2017).

Strains of ST410 are transmissable between wildlife, humans, companion animals and the environment (Falgenhauer et al., 2016a; Schaufler et al., 2015) and is already globally distributed, reported in 14 different countries. While ST131 is far more dominant globally, ST410 appears to be older than ST131 based on BEAST analysis, which puts the most recent common ancestor for ST410 at the early 1800's, while ST131's is late 1800's. Authors attribute the greater success of ST131 to its slightly superior virulence profile over ST410 (Roer et al., 2018).

7.4 ST345

Compared to the previous three strains, little is known about the prevalence of ST345. While colistin resistance is found in this strain, its geographical distribution is either grossly underrated or not highly prevalent. It seems to be associated with avian populations, present in migratory birds such as silver gull Western Australia (Ruzauskas 2016), commercial chicken meat in Brazil (Casella et al., 2017) and wild owls in South America (Fuentes-Castillo et al., 2019) and potentially its prevalence is underappreciated because of this.

7.5 ST73

There is another theory that the link between antimicrobial resistance and reproductive success may not be so straight forward. The most compelling evidence supporting this comes from an 11 year population survey across hospitals in the UK, where authors accounted for the emergence and persistence of ST131 among hospitals under the Bacteraemia Resistance Surveillance Programme of the British Society for Antimicrobial Chemotherapy (Kallonen et al., 2017). In this study, ST131 originated in 2002, and was able to displace more resistant strains that were already established in the system, such as ST88 and ST405. Crucially, ST131 did not displace other ExPEC clones, including strains like ST73 which is sensitive to most antibiotics. Instead, ST131 accounted for 20% of isolates, and this equilibrium was sustained for the duration of the study, until 2012. Kallonen et al postulated that this was likely due to the action of negative frequency dependent selection (NFDS), where different strains stably coexist in the same niche, as selection favours that which is rare. This has been observed in long term evolution studies with *E. coli*, and typically arises where more than one feeding source is available and strains do not compete for the same resource (Spencer et al., 2007). This dynamic was further explored in 2019, when McNally et al combined population frequency dynamics with pangenome analysis of the coexisting ExPEC clones (McNally et al., 2019). In clade C strains, authors noted an increased level of sequence diversity among anaerobic metabolism genes, as well as traits involved in host colonization, and hypothesized that this was evidence of adaptation in these strains. Examples included ethanolamine production and cobalamin

biosynthesis, both important for outcompeting other bacteria (McNally et al., 2016b; Winter et al., 2010). *E. coli* is a facultative organism and adaptation to the anaerobic environment has been documented experimentally (Finn et al., 2017). In a clinical setting, anaerobic adaptation among clade C ST131 is likely to facilitate enhanced persistence within the host.

8. Reason for lack of success of other clones relative to ST131

It seems there are two scenarios at play here.

8.1 Scenario 1: Resistance is paramount

Under the first scenario, the acquisition of antimicrobial resistance is crucial to the evolutionary trajectory that resulted in clade C2 ST131 and its eventual clonal expansion. Artificial selection amongst a background of antibiotics drove the lineage that acquired fluoroquinolone resistance followed by ESBL-production. This represents the highest fitness peak, or the most ideal combination of mutations and acquired traits achievable in a given environment, on a hypothetical adaptive landscape. Other peaks of lesser fitness exist, however, clade C2 emerged as the dominant form through clonal interference. We assume this is the highest peak because the addition of more resistance determinants does not seem to proliferate, most likely due to the added metabolic burden of plasmids, at least in the absence of known compensatory mutations. However this does not explain how ST131 was unable to outcompete ST73 in the British study, which remained sensitive to antibiotics but was not outcompeted by ST131 (Kallonen et al., 2017).

8.2 Scenario 2: Factors other than resistance are paramount

Alternatively, evolutionary forces other than natural and artificial selection could be at play. Rather than disruptive sweeps of clonal replacements, there could first be diversifying selection among different ST131 clades or other high risk clones, followed by coexistence in the same environment supported by NFDS. This situation would avoid a Red Queen hypothesis scenario, where microorganisms sequentially acquire new means of gaining advantages over competitors. NFDS is a stabilizing force, which favours a variant when it is rare, and leads to a balanced form of equilibrium between strains. Furthermore, other factors in addition to fitness and selection based on

antimicrobial resistance determinants are likely to play a role, such as virulence, colonization ability, transmissibility, host immunity and how these factors interact. Thus, one simple explanation is unlikely to explain an invading pathogen. Overall, it is likely that a combination of evolutionary and non-evolutionary forces are at play in creating one of the biggest biological success stories of the 2000's.

9. Conclusion

Insights from whole genome sequencing and phylogenetic studies have been invaluable in deciphering both the current population structure and the evolutionary history of ST131. ST131 is composed of five sub-clones, A, B, C0, C1 and C2. The C1 and C2 sub-lineages emerged as fluoroquinolone resistant and shortly after, C2 acquired ESBL production due to the acquisition of an IncF plasmid harbouring the $bla_{\text{CTX-M-15}}$ gene. Well defined virulence studies and large scale screening projects have built a detailed picture of the pathogenicity and antimicrobial resistance profile of the organism. However, more work remain on to be done in investigating the secrets behind ST131's phenomenal success.

Knowledge of ST131's population structure and awareness of the test strains being used as model organisms for wet lab experiments are critical. Care is needed to ensure that proper controls are in place when inferring the role of precise mutations among strains with different genetic backgrounds, and should be tested in biologically meaningful environments, rather than solely in rich lab media. While many fitness inferences have been devised by comparing different ST131 strains as outlined in a recent review (Whitmer et al., 2019), more in depth analysis is required through the development and testing of isogenic mutants of ST131 to identify the precise role particular mutations and genes have had on the fitness and overall evolution of the organism. Additionally, horizontal gene transfer, and the rate at which it occurs, introduces unpredictability to the system, and our understanding of these two factors is key to getting ahead in the current arms race *status quo*, as we still do not have a working model for the controlled study of HGT. This is a glaring omission as it is bacteria's greatest innovation.

The fitness of an organism comprises of the sum total of all contributing phenotypes in the cell, with each variable likely contributing to the organism's success. We know the combination of the ST131 genetic background, armed with an IncF plasmid containing $bla_{CTX\text{-}M\text{-}15}$, in a sub-clone that developed compensatory mutations mediating resistance to fluoroquinolones was a recipe for success. Nevertheless, in an environment with increasing demands on antibiotic use, clinicians and evolutionary biologists must be informed of the probabilities of potentially succesful evolutionary trajectories. Overall, ST131 is an excellent real-world model to study of the emergence of a superbug. However, more needs to be done to understand the evolution of these multidrug resistant organisms to guide rational drug design, inform antibiotic stewardship programs and control the evolution of other nascent emerging pathogens.

10. Compliance with Ethical Standards

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10.2 Conflict of interest

JP and TF have no conflicts of interest relevant to the content of this article.

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