Profiling the plasmid conjugation potential of urinary Escherichia coli

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Abstract

Escherichia coli is often associated with urinary tract infection (UTI). Antibiotic resistance in E. coli is an ongoing challenge in managing UTI. Extrachromosomal elements – plasmids – are vectors for clinically relevant traits, such as antibiotic resistance, with conjugation being one of the main methods for horizontal propagation of plasmids in bacterial populations. Targeting of conjugation components has been proposed as a strategy to curb the spread of plasmid-borne antibiotic resistance. Understanding the types of conjugative systems present in urinary E. coli isolates is fundamental to assessing the viability of this strategy. In this study, we profile two well-studied conjugation systems (F-type and P-type) in the draft genomes of 65 urinary isolates of E. coli obtained from the bladder urine of adult women with and without UTI-like symptoms. Most of these isolates contained plasmids and we found that conjugation genes were abundant/ubiquitous, diverse and often associated with IncF plasmids. To validate conjugation of these urinary plasmids, the plasmids from two urinary isolates, UMB1223 (predicted to have F-type genes) and UMB1284 (predicted to have P-type genes), were transferred by conjugation into the K-12 E. coli strain MG1655. Overall, the findings of this study support the notion that care should be taken in targeting any individual component of a urinary E. coli isolate’s conjugation system, given the inherent mechanistic redundancy, gene diversity and different types of conjugation systems in this population.

DATA SUMMARY

This study uses the urinary E. coli isolates, sequence reads, and contigs first published in Garretto et al. 2020 (Table S1). Assembled whole genome sequences (WGS) for these isolates were downloaded from NCBI BioProject PRJNA316969. Raw sequence reads for transconjugant K-12 respectively carrying pU1223 (SRR15011177) and pU1284 (SRR15011176) were submitted to GenBank.

INTRODUCTION

Escherichia coli is linked to urinary tract infection (UTI) [1–3]. Because the key genetic determinants of E. coli pathogenicity in the urinary tract remain unknown, management is a challenge [4–6]. This clinical dilemma is compounded by antibiotic resistance [7, 8]. Genes that encode antibiotic resistance are often carried by plasmids, genetic elements that can propagate and retain genes not only for resistance but also for virulence and fitness [8–10]. Due to their variable genetic composition, plasmids can be classified by incompatibility (inc) grouping, which denotes the inability of two plasmids to be maintained in the same host [11–13]. A prime example of clinically relevant plasmids in E. coli are the F plasmids, characterized by the inc loci incFI and/or incFII [14, 15]. F plasmids are widespread, heterogenous, stable and generally mobile [8, 10, 16]. F plasmids have frequently been found in urinary E. coli, specifically in extended-spectrum β-lactamase (ESBL)-producing E. coli isolated from urinary infection [17]. Also, the uropathogenic E. coli (UPEC) strain UTI89 harbours an F plasmid hybrid – pUTI89 – and its loss results in a significant decrease in pathogenicity in a mouse model during early infection [18]. The pUTI89 plasmid has been noted to carry pathogenesis genes in addition to a conjugation system, which is key for plasmid mobility [6, 16, 19].
Impact Statement

In this study, we sought extrachromosomal elements – plasmids – in the draft genomes of 65 *Escherichia coli* strains isolated from bladder urine of adult women with and without urinary tract infection-like symptoms. We profiled conjugation systems that *E. coli* uses to transfer genes between cells. We performed this analysis because others had proposed to target conjugation components as a strategy to curb the spread of plasmid-borne antibiotic resistance. Our findings suggest caution in targeting any individual component of a urinary *E. coli* isolate’s conjugation system, given the inherent mechanistic redundancy, gene diversity, and different types of conjugation systems in this population.

Conjugation is the cell-to-cell translocation of genetic information assisted by conjugative machinery – conjugative pilus, DNA processing enzymes (relaxosome), mating pair stabilization proteins and the type IV secretion system (T4SS) [16, 20]. The prototypical conjugation system in *E. coli* is the F-type system, which uses transfer (tra) genes [10, 21, 22]. The transfer operon is composed of genes that are considered to be essential for conjugation (traA, traB, traK, traC, traW, trbc, traE, traL, traH, traG, traU, traI, traM, traD) for F plasmid transfer and, in some cases, more than a dozen accessory genes [19, 20, 22]. Because expressing conjugation machinery is an expensive endeavour, it is repressed by the fertility inhibition (FinOP) system that targets the transcription regulator TraJ [23]. TraJ upregulates the transfer operon, resulting in the expression of components involved in forming the relaxosome (traD, traI, traM, traY), conjugative pilus (traP, traE, traL, traC, traW, trbc, traF, trbr), mating pair stabilization (traN, traG, traU) and T4SS core proteins (traB, traK, traV) [16, 20, 21, 23]. The transfer operon also can encode proteins that defend against excessive conjugation with the same or similar plasmids, either by surface exclusion (TraT) or entry exclusion (TraS) [23, 24]. F plasmids are the typical group associated with the F-type conjugation system [16].

Functional and structural relatives of the F-type transfer system exist, such as the P-type VirB/VirD4 system, which also utilizes a T4SS for conjugation and has been extensively studied in *Agrobacterium tumefaciens* [16, 20, 25]. Components consist of the type IV coupling protein (VirD4), inner membrane complex (VirB4), outer membrane complex (VirB7, VirB9, VirB10), conjugative pilus (VirB2 pilin), transglycosylase (VirB1) and pilus-tip protein (VirB5) [16, 26]. The plasmids RP4, R388 and pKM101 are well-studied *E. coli* plasmids associated with a P-type system [27–29]. The VirB/VirD4 system has been implicated not just in intra-species genetic exchange, but also in inter-species and even inter-kingdom conjugation [30, 31]. Likely due to the constant genetic exchange in plasmids that results in genomic heterogeneity, bacteria may carry plasmids that borrow components from different conjugation systems [16, 25].

Conjugation is a potential target in curtailing the spread of antibiotic resistance in the clinical setting [26, 32]. Hypothetically, blocking key transfer functions by conjugation inhibitors (COINs) could decrease plasmid exchange and, by extension, decrease propagation of bacterial genes for antibiotic resistance, virulence and fitness [33–37]. It has been noted that COINs should ideally not inhibit bacterial growth and be used in conjunction with antibiotics [32]. However, deployment of such a strategy requires further considerations [26, 32]. Outside of identifying genes essential for conjugation and determining how to mechanistically target them, we must know how a particular bacterial population depends on a given conjugation system. Targeting a conjugation component would be less effective if this factor was not abundant in a population or if this factor could be replaced by another component. Furthermore, if there were multiple distinct conjugation systems in a population, the targeting of one system might simply lead to the selection and propagation of the other [38, 39]. In considering conjugation as a viable target for inhibition in the clinical setting, we must first understand in more detail the conjugation systems in clinical bacterial isolates in a given niche.

In this study, we profile F-type and P-type conjugation genes in 65 urinary *E. coli* isolated from catheterized bladder urine of adult women with or without UTI-like symptoms [5]. Plasmids were present in 84.6% (N=55/65) of urinary *E. coli* isolates, and the majority included F-type conjugation genes with the minority having the P-type conjugation genes. To validate conjugation of these urinary plasmids and viability of the F-type and P-type systems, we transferred by conjugation the plasmids from two isolates, UMB1223, which has F-type genes, and UMB1284, which has P-type genes, into the *E. coli* K-12 strain MG1655. Multiple types of conjugation loci exist in urinary *E. coli*. Overall, our investigation finds that conjugation systems are common in urinary *E. coli* and that conjugation genes are diverse in number and composition. This knowledge is important to understanding conjugation in the clinical setting and how this process may be targeted.

METHODS

This study uses the urinary *E. coli* isolates, sequence reads and contigs first published by Garretto et al. in 2020 (Table S1, available with the online version of this article) [5]. These isolates were recovered from urine samples collected from adult women with and without UTI-like symptoms via transurethral catheterization during several Institutional Review Board-approved studies at Loyola University Chicago (USA) (LU203986, LU205650, LU206449, LU206469, LU207102 and LU207152) and the University
of California San Diego (USA) (170077AW). Assembled whole-genome sequences (WGSs) for these isolates were downloaded from National Center for Biotechnology Information (NCBI) BioProject PRJNA316969.

To identify known plasmid inc loci, E. coli WGS fasta files were scanned using PlasmidFinder v2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) using the Enterobacteriaceae database with a threshold of 95% identity and a minimum 60% coverage (Table S2) [13]. The genomes were binned into a plasmid group associated with their inc genes via exclusion criteria: genomes with incF genes were placed in the IncF group, genomes with col but no incF genes were placed in the Col group, and genomes with inc genes that were neither incF nor col inc genes were in the Inc- various group. The E. coli plasmid literature was reviewed for genes used in plasmid conjugation, primarily from the F-type transfer and P-type VirB-VirD4 systems (Tables S3 and S4) [20, 23]. NCBI Local blast v2.11.0 was used to query this initial set of conjugation genes in the 65 urinary E. coli draft genomes [40, 41].

Conjugation was utilized as the method to transfer plasmids from urinary E. coli isolates to a laboratory E. coli K-12 strain (MG1655 with a chloramphenicol-resistance cassette) that possessed a chloramphenicol selection marker [42]. To attempt conjugation, we searched for compatible antibiotic-resistance traits in urinary isolates (potential conjugation donors), CamS and resistance to one of the following: kanamycin, spectinomycin, tetracycline, ampicillin. Urinary E. coli isolates with identified plasmids, the appropriate resistance markers and carrying transfer genes were grown on various antibiotic plates to assess their selection marker profile. Lysogeny broth (LB) agar plates (1.0% bacto tryptone, 1.0% yeast extract, 0.5% NaCl, 1.5% agar) were supplemented with these respective antibiotics: ampicillin (Amp, 100 μg ml⁻¹), chloramphenicol (Cam, 25 μg ml⁻¹), kanamycin (Kan, 40 μg ml⁻¹), spectinomycin (Spec, 100 μg ml⁻¹) or tetracycline (Tet, 15 μg ml⁻¹) (Table S5). Isolates that grew on an antibiotic had their sequences reviewed for antibiotic-resistance genes in their contigs, which also were compared to plasmid entries in the NCBI nr/nt database. Urinary E. coli plasmid donor candidates were filtered on the following basis: they could grow on an antibiotic selection marker, they carried a gene that was predicted to encode antibiotic resistance, this resistance gene was on a predicted plasmid, and they had evidence of near complete F-type or P-type operons (Table S6) [16].

Two urinary E. coli (UMB1223, UMB1284) met the criteria and were used as plasmid donors; UMB0939 was used as a negative control, as it met all the criteria except identifiable conjugation genes. Conjugation in E. coli was performed as described elsewhere (Table S6) [42]. K-12 transconjugants carrying the urinary plasmids were able to grow on double chloramphenicol and tetracycline LB agar plates. For DNA extraction, bacteria were grown as liquid cultures in LB (with chloramphenicol and tetracycline as selection markers) at 37°C in a shaking incubator for 12h. Whole-genome DNA was extracted using a Qiagen DNEasy UltraClean microbial kit using the manufacturer's standard protocol. Plasmid DNA was extracted using a Qiagen large-construct kit using the manufacturer's standard protocol. A Qubit fluorometer was used to quantify the DNA concentration in the obtained genomic and plasmid DNA preparations. Nextera XT DNA library preparation kit was used to make DNA libraries, which were sequenced on the Illumina MiSeq platform using the MiSeq reagent kit v2 (500 cycles) at the Loyola University Chicago’s Genomics Facility (Maywood, IL, USA). Raw sequence reads for transconjugant K-12 respectively carrying pU1223 (accession no. SRR15011177) and pU1284 (accession no. SRR15011176) were submitted to GenBank. The raw sequence reads from the whole-genome extraction were assembled using plasmids.pades.py of SPAdes v3.12 with k values of 55, 77, 99, 127 and the only- assembler parameter [43]. Assemblies were renamed via a bash script and contigs less than 500 bp were removed via Bioawk. Plasmidic assemblies were queried against the nr/nt database via NCBI’s web blast and hits were binned as either E. coli plasmid or chromosome [40, 41]. A curated plasmidic assembly was made using only contig hits to plasmid sequences by pruning contigs with homology to chromosomal entries in the nr/nt database.

NCBI Local blast v2.11.0 was used to query conjugation genes, as described above, in the plasmidic assemblies for plasmids pU1223 and pU1284 [40, 41]. pU0928 and pU1284 were annotated via Prokka v1.13.5 and the location of F-type and P-type conjugation genes was identified using the genome viewer function of Geneious Prime v2021.1prokk [44, 45]. Predicted genes in these loci were queried against the NCBI nr/nt collection via blast [40, 41]. Conjugation genes in pU1223 and pU1284 not included in our initial query list were concatenated into a new query list and all 65 urinary E. coli draft genomes were queried for these additional genes. Hits with a query coverage over 85% and sequence identity over 90% were recorded as present. We designated these parameters to have a high homology threshold while accounting for reported variable regions in conjugation genes [20]. The proportion of all genes queried, including those in the initial scan and those newly identified in pU1223 and pU1284, was calculated for all urinary E. coli genomes.

RESULTS
Sixty-five urinary E. coli draft genomes were queried by PlasmidSPAdes to identify plasmid inc genes (Table S2). These 65 strains were isolated by our team via catheterization and, thus, are members of the bladder microbiota, which differs from the urethral microbiota [46] that is also present in voided urine samples. For each genome sequence, we also have symptom data from the participant. To facilitate plasmid group analyses, we binned isolates with incF genes into the IncF group, isolates with col genes but no incF genes in the Col group, and isolates with inc genes that were neither incF nor col into the Inc- various group. We performed an initial query utilizing F-type and P-type genes on the 65 urinary E. coli draft genomes (genes queried are listed in Tables S3 and S4) [16, 20]. Genes previously associated with F plasmid transfer, most notably
tra genes, were in relatively high proportion in draft genomes with F-type genes (Fig. 1). To validate plasmid conjugation in these isolates, we respectively chose two urinary E. coli isolate predicted to have a F-type system (UMB1223) and P-type system (UMB1284) [16]. UMB1223 and UMB1284 could grow on tetracycline plates and their tetracycline-resistance gene was localized to a putative plasmid sequence within the genome assembly (Table S5). Our initial gene query utilizing reference conjugation genes indicated that UMB1223 had 23 tra genes in a putative plasmid sequence, while UMB1284 had 5 virB genes in addition to 2 tra genes (Table S6). To confirm conjugation, the genetic content in the MG1655 transconjugants was extracted, sequenced and submitted to GenBank (K-12 with plasmid from UMB1223 under accession no. SRR15011177, K-12 with plasmid from UMB1284 under accession no. SRR15011176). The raw sequence reads were assembled into plasmid contigs (pU1223 and pU1284, respectively) and analysed. We verified the presence of a tetracycline-resistance cassette, incF loci and conjugation genes like those present in the original (donor) urinary isolates (compare Table 1 to Table S6).

We reviewed the contigs in pU1223 and pU1284 for the presence of conjugation loci, including genes we may not have identified in our initial query using conjugation gene references (Tables S3 and S4). The conjugation locus in pU1223 consisted of 39 ORFs that included the transfer genes we had profiled in the parent isolate UMB1223; it also included variants of transfer genes not initially identified, two transposons and four hypothetical genes (Fig. 2, Table S7). The transfer locus was organized in the prototypical manner of a F-type transfer operon, with finO upstream and both traJ and traM downstream of the rest of the tra genes, plus the presence of all tra genes essential for conjugative transfer [20, 21]. The operon contained 17 non-essential transfer genes, in addition to two transposon sequences mid-operon, plus four hypothetical (HP) coding regions flanked by transfer genes. HP_1 was 109 amino acids long and was not homologous to any entries in the NCBI database or known protein domains. HP_2 had some homology to the neighbouring traC, while HP_3 and HP_4 had homology to the neighbouring traJ. Seven of the transfer genes identified in pU1223 were variants of the reference sequences we initially used to carry out the profile and were not initially identified because they failed to meet our threshold relative to the queries used; all these variants had high homology to transfer genes in GenBank (Table S7). pU1284 contained the P-type virB-virD4 operon in one contig and a locus with F-type genes in another contig (Fig. 2). Except for virB3 and virB7, pU1284 had all VirB-VirD4 genes in the same locus with a prototypical sequential organization (Table S8). Some of the vir components missed in our initial profile were present in the operon as a variant of our initial query sequence. We also identified three novel genes with T4SS homology upstream of virD4, between virB6 and virB5. These T4SS ORFs had high sequence identity

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**Table 1. Overview of urinary plasmids sequenced from E. coli K-12 transconjugants**

| Background strain | Urinary E. coli plasmid donor | Urinary plasmid replicon | Tc resistance gene in plasmid | Conjugation genes identified |
|-------------------|--------------------------------|--------------------------|-------------------------------|------------------------------|
| MG1655            | UMB1223                        | col156, incFIA, incFIB, incFIII | Yes                           | F-type                       |
| MG1655            | UMB1284                        | incFIA, incFII, incX       | Yes                           | P-type, F-type               |

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Fig. 1. F transfer genes in sequences with evidence of an F-type system. Transfer genes were identified in urinary E. coli genomes. Genomes with evidence of an F-type system were further analysed for the presence of transfer genes required for conjugation and, thus, which could most likely succeed in transferring their plasmid by conjugation to E. coli K-12.
and query coverage to entries associated with conjugative and T4SS function in GenBank (Table S8). T4SS_1 had a conjugal protein domain (PRK13893, pfam07424) like that in TrbM. T4SS_2 had protein domains of the Cag pathogenicity island proteins (pfam13117). T4SS_3 had the TrbL protein domain associated with conjugal proteins, in addition to a VirB6-like domain and conjugal protein domain like that in TrbL (pfam04610, COG3704, PRK13875). A hypothetical gene (hypo_vir) was also found flanked by virB8 and virB6, where the missing virB7 is normally located. In addition to the virB-virD4 operon, pU1284 had a locus in another contig with transfer genes including traD, traT, traS, traG, traH and a hypothetical ORF (HP_5) (Table S8). HP_5 had no distinct homology to entries in the NCBI database, but was predicted to have multiple transmembrane domains.

We profiled all the urinary E. coli genomes using the newly identified ORFs in the putative conjugation regions from pU1223 and pU1284 (Fig. 3, Table S9). We estimated that plasmids are present in 84.6 % (N=55/65) of these urinary E. coli isolates; most draft genomes had incF genes (81.8 %, N=45/55). F-type conjugation genes were present in 72.7 % of plasmids (N=40/55), all but one had incF genes (Table S9). No conjugation genes were predicted in the genomes from the Col group (Table S9).

One urinary E. coli draft genome from the Inc- various group had 28 tra genes (Table S9). Urinary E. coli with no inc gene hits did not have F- type nor P- type conjugation genes (Table S9). Nearly all F- type genes queried were present in draft genomes from the IncF group, with a mean gene hit count of 27.1 genes per genome (Fig. 3). Multiple sequence variants were identified for traJ, traS and traY. The genes trbP, trwB, trwC and trwM were not found in any of the urinary E. coli genomes (Table S9).

Type-P genes, consisting of the VirB-VirD4 system, were present in 18.2 % (N=10/55) of the urinary E. coli isolates with putative plasmids (Table 2). Five genomes (UMB0931, UMB1284, UMB1727, UMB6721, UMB7431) were predicted to encode vir genes like those in pU1284, encompassing a near complete VirB-VirD4 operon. Urinary E. coli genomes with a near complete VirB-VirD4 operon (including pU1284) had the gene incFII. Except for UMB1348, draft genomes with a small number of P-type genes did not have the gene incFII but rather incFI (UMB6655, UMB6890) or incXI (UMB1228) (Table 2). Six urinary E. coli genomes (UMB0931, UMB1284, UMB1727, UMB6721, UMB7431, UMB6653) were predicted to encode the three novel T4SS-like ORFs.

**DISCUSSION**

Urinary E. coli are associated with UTI [1–4, 47, 48]. In urinary isolates of E. coli, virulence factors and antibiotic-resistance cassettes are often retained and transmitted by conjugal plasmids [10, 49]. F plasmids have been reported previously as present in the urinary tract, both in E. coli strains isolated from patients with UTI diagnoses and in an F-like plasmid hybrid in the uropathogenic E. coli (UPEC) strain UTI89 [6, 17, 18]. Here, we have examined a representative set of urinary E. coli genomes from the bladders of adult women with and without UTI symptoms, providing evidence and investigation of the plasmid conjugation genes.

Conjugation components have been considered as targets to decrease the spread of plasmid-borne traits in the clinical setting [26, 32]. For this strategy to be viable, we must understand the conjugation systems in the niche of interest. The urinary
Plasmid pU1223 had the F-type conjugation system, the most common conjugation system in the collection of urinary E. coli examined here (Fig. 2, Tables S7 and S8). The conjugation operon in pU1223 had all genes deemed ‘essential’ in an F-type system, in addition to 17 accessory genes; however, not all of the other urinary E. coli draft genomes followed this pattern [16, 20]. While genes such as traA, traH, traG, traI and traD were detected in more than 90% of draft genomes with F-type genes, there was no single gene present in all these genomes (Figs 1 and 3). We could infer that even if we targeted one of these high proportion components, a minority of urinary E. coli could potentially be unaffected.

Plasmid pU1284 had two conjugation loci, one a virB-virD4 operon and the other an F-type transfer locus (Fig. 2, Table S8). We identified VirB-VirD4 genes in ten urinary E. coli draft genomes (Tables 2 and S9). Broadly, these urinary E. coli isolates with P-type genes fell into two groups. Plasmids with the first group had a near complete VirB-VirD4 operon similar to pU1284; all of these urinary E. coli genomes had the incFII gene. The second group had VirB-VirD4 genes that differed from those in pU1284; all but one lacked the incFII gene (Table 2). Urinary E. coli plasmids with P-type genes may represent a conjugative plasmid subpopulation; within this subpopulation, there may be further subgroups (i.e. those like pU1284 and those unlike pU1284) [14, 38, 50]. The presence of a minority of plasmids with a P-type conjugation system presents a troublesome scenario for targeting conjugation in this niche [14]. If we employ antibiotics against F-type genes, potentially we indirectly favour the propagation of plasmids with the P-type system.

Fig. 3. Proportion of tra genes in the urinary E. coli plasmid group. The urinary E. coli genomes were scanned for all F-type genes, including variants and newly identified genes in pU1223 and pU1284. Urinary E. coli isolates with incF loci had a higher proportion than other plasmid groups in nearly every gene tested.
| Strain  | Incompatibility loci | T4SS_1 | T4SS_2 | virD4 | virD4_2 | virB1_2 | virB11_2 | virB10_2 | virB9_2 | virB8 | virB7 | virB6_2 | virB6 | T4SS_3 | virB5 | virB4 | virB3 | virB2 | virB2_2 | virB1_2 | virB1 |
|---------|----------------------|--------|--------|-------|--------|--------|--------|--------|--------|------|------|--------|------|--------|------|------|------|------|--------|--------|------|
| UMB0931 | incFIA, incFII, incFII, incX4 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
| UMB1284 | incFIA, incFII, incX4 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
| UMB1727 | incFIA, incFII, incFII, incI2, incX4 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
| UMB6721 | incFIA, incFII, incFII, incFII, incI2, incX4 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
| UMB7631 | incI2, incX1, incX1, incX4 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
| UMB6053 | incFIA, incFII, incI2, incX4 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
| UMB6055 | incFIA, incFII, incI2 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
| UMB1348 | incFIA, incI2, incX1 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
| UMB1228 | incX2 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
| UMB690 | incI2 | +      | +      | +     | +      | +      | +      | +      | +      | +    | +    | +      | +    | +      | +    | +    | +    | +    | +      | +      | +    |
A further layer to the diversity of conjugation systems in urinary *E. coli* is that pU1284 not only had a P-type operon, but also an F-type locus with the F-type conjugation genes *traD*, *traG* and *traH* involved in the relaxosome, mating pair stabilization and pilus retraction, respectively (Fig. 2, Table S8). The F-type locus also had *traT* and *traS*, both involved in exclusion of invading plasmids in surface and entry exclusion, respectively [24, 52]. In addition to UMB1284, five urinary *E. coli* (UMB0931, UMB1727, UMB6721, UMB6653 and UMB7431) had a VirB-VirD4 system and different combinations of F-type transfer genes (Tables 2 and S9). These combinations of P-type and F-type genes could be linked to different environmental and evolutionary histories in the urinary tract [14, 16, 53]. This mosaic structure also highlights how urinary *E. coli* plasmids, such as F plasmids, can be genetically promiscuous [14]. Even if we target a conjugation system, this could be neutralized by the re-shuffling of genetic content in these plasmids to generate mosaic conjugation loci.

An important factor to consider is that our urinary *E. coli* isolates were collected from the bladders of different participants [5]. Potentially, the diversity of conjugation genes in the urinary tract of a single person is much lower [54]. Notwithstanding, we postulate that whole-genome sequencing and conjugation gene profiling would be key to the use of antibiotics that target conjugation components, especially in combination with traditional antibiotics [32]. Plasmid genetic variation in patients presents its own challenges, however, as targeting a single conjugation component may only be effective in a subset of cases. Overall, our evidence supports the concept that conjugation systems are common in urinary *E. coli*, especially in genomes with *incF* genes, and that these genes are diverse in number and composition [10]. The F-type system is the most common, but a minority of urinary *E. coli* had evidence of a P-type system [16]. Furthermore, urinary *E. coli* like UMB1284 appear to have supplemented their P-type system with some F-type genes, including a plasmid exclusion gene.

Conjugation components have been proposed as targets to limit the spread of plasmid-borne traits, such as antibiotic resistance [26, 32]. Targeting of a specific conjugation component should be considered in the context of (i) how frequent is that component in the plasmid population, (ii) how vital that component is to the conjugation system, and (iii) whether another conjugation system in the population could be uncathed. Modularity and redundancy is inherent to conjugation systems in bacteria and targeting isolated components could be ineffective or worse negatively affect the patient [10, 14, 16]. For example, by deploying antibiotics that target the F-type system, we may inadvertently increase the frequency of P-type conjugation, which could alter plasmid and microbiome dynamics [32]. The presence of different conjugation systems, frequency of individual conjugation genes, and combinations of these genes require further attention, as they may be key to plasmid exchange in the urinary tract. Conjugation systems in clinical isolates must be well characterized as part of our efforts to restrict the spread of plasmid-borne antibiotic resistance.

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