Transcriptomic Analysis of *Escherichia coli* CFT073 Colonized in the Mammalian Intestine

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ABSTRACT

Uropathogenic *Escherichia coli* (UPEC) strain CFT073 is a common cause of hospital acquired urinary tract infections (UTIs) and has been shown to switch metabolic preferences from gluconeogenic to sugar substrates when colonized in the urinary and intestinal tracts, respectively. Interestingly, CFT073 also exhibits a quiescent phenotype when plated below a threshold of 10^6 CFUs on minimal media *in vitro*, suggesting a link between the recurrence of CFT073-related UTIs and the ability to remain colonized. This research project used the streptomycin-treated mouse model to first acquire total cellular RNA from *E. coli* CFT073 colonized in mucosal layer of the mouse cecum. Next, we evaluated whether genes involved in regulation the quiescent phenotype in *E. coli* CFT073 grown *in vitro* also play a role in colonization. Lastly, we provide some details of an algorithm-based approach to the analysis of RNA Sequencing (RNA-seq) datasets generated using two mechanistically different sequencing strategies to identifying primary transcripts.

1. Introduction

In the United States, uropathogenic *Escherichia coli* (UPEC) causes approximately 80% of urinary tract infections (UTIs) experienced by women (Nicolle et al., 2002). Moreover, the source of recurrent UTIs, which may lead to cystitis, has been traced most frequently back to the UPEC responsible for the initial infection (Russo et al., 1995). *E. coli* strain CFT073, the prototypical UPEC, uses sugars during colonization of the mammalian intestine and traverses the urethra to gain access to the bladder where it switches to metabolism of gluconeogenic substrates (Alteri et al., 2009, Meador et al., 2014). Once colonized in the bladder, CFT073 can subsequently ascend the ureters and cause acute pyelonephritis in the kidneys (Mobley et al., 1990). Data describing mechanisms utilized by enteric bacteria to compete for preferred nutrients in the intestine is insufficient.

Recent reports suggest that antibiotic resistant quiescent intracellular reservoirs (QIRs) of UPEC cells mediate recurrent UTIs (Kerrn et al., 2005, Silverman et al., 2013). It has been shown that seeding CFT073 at $\leq 10^6$ CFUs on glucose M9 minimal agar causes the cells to enter a state of quiescence, a phenotype which can be disrupted by introducing

mutations in 6-phosphogluconate dehydrogenase (*gnd*), glutamate dehydrogenase (*gdhA*), pyruvate kinase (*pykF*), lysine permease (*lysP*), and glucose-6-phosphate dehydrogenase (*zwf*) (Leatham-Jensen et al., 2016). The onset of disease caused by urinary tract pathogens is contingent upon their initial colonization of the intestine. Therefore elucidating UPEC mechanisms of intestinal colonization is critical to the development of therapeutic approaches to reduce the potential dissemination of infections to nearby tissues. However, the relationship between intestinal colonization and cellular quiescence has not been sufficiently studied. Specifically, it is unknown whether the same genes that regulate the quiescent phenotype, observed when CFT073 is seeded on glucose M9 minimal media, also play a role in colonization of the streptomycin-treated mouse model.

To begin characterizing the gene expression profile of *E. coli* CFT073 colonized in the mammalian intestine, *E. coli* CFT073 was colonized alone or co-colonized with isogenic mutants exhibiting a defect in a pathway regulating quiescence *in vitro*. Strain populations were monitored by fecal plate counting, and total cellular RNA was extracted from cecal mucus samples collected immediately following euthanization using a modified hot phenol extraction protocol. Currently, we are using high-resolution promoter mapping to annotate differential ribonucleic acid sequencing (dRNA-seq) data generated from the extracted bacterial RNAs.

2. Experimental Details

Bacterial strains. All strains used in this study are listed in Table 1. *E. coli* CFT073 is a sequenced UPEC prototype that colonizes the gastrointestinal tract. It was originally isolated from a patient with acute pyelonephritis and found to exhibit hemolytic activity on blood agar plates (Mobley et al., 1990). In order to colonize streptomycin-treated mice, all strains were made streptomycin resistant (Str^r). To facilitate fecal plate counting of individual populations during co-colonization experiments, all St^r strains were made either nalidixic acid resistant (Nal^r) by spontaneous mutation or Kanamycin resistant (Km^r) by random insertion of the mini-Tn*5* Km transposon (Meador et al., 2014, Leatham-Jensen et al., 2016, Moller et al., 2003).

Table 1

***E. coli* Strain**	**Relevant genotype or description**	**Source or reference**
CFT073 Str^r Nal^r	Spontaneous Str^r Nal^r	(Meador et al., 2014)
CFT073 Str^r mini-Tn5 Km::*gnd*	Mini-Tn5 Km 6-phosphogluconate dehydrogenase mutant of CFT073 Str^r	Jodi Camberg
CFT073 Str^r mini-Tn5 Km::*pykF*	Mini-Tn5 Km pyruvate kinase mutant of CFT073 Str^r	Jodi Camberg
CFT073 Str^r mini-Tn5 Km::*gdhA*	Mini-Tn5 Km glutamate dehydrogenase mutant of CFT073 Str^r	Jodi Camberg
CFT073 Str^r mini-Tn5 Km::*zwf*	Mini-Tn5 Km glucose-6-phosphate dehydrogenase mutant of CFT073 Str^r	Jodi Camberg
CFT073 Str^r Δ*lysP*::Km	*lysP* Deletion mutant of CFT073 Str^r	Jodi Camberg

Streptomycin Mouse Model. The addition of streptomycin-sulfate in the drinking water provided to conventional mice selectively removes facultative anaerobes, increases mucosal carbohydrate availability, and permits the experimental association of *E. coli* strains for which the genome sequences are available (Wadolkowski et al., 1988, Hentges et al., 1984, Ng et al., 2013). This non-invasive mechanism of antibiotic treatment has been used extensively to provide complete control over the facultative anaerobe population colonized in the mucosal layer of the mouse gastrointestinal tract, specifically the cecum (Conway and Cohen, 2007). Briefly, 6-week-old mice (male, CD-1) were obtained from Charles River Laboratories, acclimated to the housing environment for 72 hrs., then separated into individual cages and provided with sterile filtered streptomycin-sulfate treated water (5 g/Liter) for 24 hrs. Following streptomycin-sulfate treatment, both food and water were removed for 14 hrs. and then mice were fed approximately 10^9 CFU of *E. coli* strain(s) in 1 ml of 2% sucrose (day 0). After consuming the bacterial suspension, food and water (5 g/Liter streptomycin-sulfate) were given to the mice ad libitum for the duration of the experiment.

Plate Counting. Antibiotic resistance to either St^r and Nal^r or St^r and Km^r was used to differentiate the strains in fecal plate counts. Feces were diluted 1:10 (w:v) in 1% tryptone, serially diluted, and plated on lactose-MacConkey agar supplemented with the appropriate antibiotics at the following working concentrations: streptomycin sulfate (100 mg/ml), nalidixic acid (50 mg/ml), and kanamycin (40 mg/ml). Each colonization experiment was repeated, and the values for the six mice (or more) were averaged. The $\log_{10}$ CFU/g feces was determined for each strain at each time point (± the standard error of the mean). A fold difference in CFU/g feces $\geq 10^1$ between strains was statistically significant.

RNA isolation. Following euthanization, approximately 100 μL of cecal mucus was gently scraped into an equal volume of ice-cold RNA stabilization buffer (3.53 M Ammonium sulfate, 17 mM Sodium citrate, 13 mM EDTA, pH 5.2) and pipetted up and down to mix and prevent RNA degradation. Cells were separated from mucus by centrifugation at 14,000 x g for 5 min. Total RNA cellular was isolated from CFT073 St^r Nal^r colonized in the mucus samples using a modified hot phenol protocol (Ares, 2012).

Briefly, 400 μL boiling lysis buffer (2% SDS, 10 mM EDTA, 10 mM Tris) was pipetted on to cell pellets immediately causing lysis. Phenol:chloroform:isoamyl alcohol (PCA, 25:24:1) was added to the lysate, vortexed to emulsify, and incubated at 65°C for 10 min. This was followed by two PCA extractions and two chloroform washes. After the second wash, RNA in the aqueous phase was taken through the Quick-RNA™ Soil/Fecal Microbe Microprep protocol. Contaminating DNA was removed by digestion with Turbo DNase (Invitrogen). The concentration and integrity of RNA was determined by spectrophotometry and agarose gel electrophoresis, respectively. RNA samples were stored at -70°C.

RNA-Seq and High Resolution Promoter Mapping. Total RNA was shipped on dry ice to vertis Biotechnlogie AG (Freising, Germany) for cDNA library preparation and sequencing on an Illumina HiSeq2000. For differential RNA-seq (dRNA-seq) analysis, one portion of each RNA sample was fragmented, and modified on the 3'- and 5'- end with a poly(A) tail and RNA adapter, respectively. Reverse transcriptase and poly(dT) primer were used to in first-strand cDNA synthesis, and a barcoded TrueSeq adapter was incorporated during the synthesis of the second strand. A second portion of each RNA sample was similarly fragmented as the first, then subject to treatment with terminator exonuclease (TEX), which selectively digests RNA with 5' monophosphate ends thereby enriching for 5'-triphosphorylated ends characteristic of transcription start sites (TSS) at promoters. Following digestion, TEX treated RNA's were tailed and ligated as described above. To begin constructing a comprehensive and high-resolution promoter map of *E. coli* CFT073 using differential dRNA-seq methodology, the following criteria were applied to identify statistically significant promoters: (1) consensus among biological replicate data sets, (2) sequencing read enrichment facilitated by terminator exonuclease (TEX); (3) Increased sequencing reads in the TSS+9 bp region relative to the TSS-9 bp region in untreated (coverage) data sets, and (4) promoter motif analysis.

3. Results

While facultative anaerobes have previously demonstrated to prevent colonization of enterohemorrhagic *E. coli* (EHEC) strain O157:H7, ureopathogenic *E. coli* CFT073 (UPEC) and enteropathogenic *E. coli* E2348/69 (EPEC) both grew from low to high numbers in the mouse intestine when associated on day 10 of streptomycin mice pre-colonized with commensal strains *E. coli* HS and *E. coli* Nissle 1917 (Maltby et al., 2013; Leatham et al., 2009). Similarly, our data support that *E. coli* CFT073 can successfully grow from low to high numbers when 10^5 CFUs are associated in the streptomycin-treated mouse in the absence of native facultative anaerobes (Fig 1A).

We found that when 10^5 CFU each of CFT073 wild-type and CFT073 *pykF* were fed simultaneously, CFT073 wild-type colonized at ~10^9 CFU for the duration of the experiment, whereas CFT073 *pykF* initially grew to ~10^8 CFU after 24 hrs., but decreased to ~10^6 CFU by day 15 (Fig. 1B). Similarly, CFT073 *lysP* initially grew to ~10^7 CFU after 24 hrs., but also decreased to ~10^6 CFU by day 15 (Fig. 1D). Neither CFT073 *gdhA, gnd, zwf* showed

significant colonization defects when 10^5 CFUs of each was simultaneously colonized with 10^5 CFUs of CFT073 wild-type (Fig. 1C, E, F).

Using our modified version of a hot phenol extraction protocol in combination with the Quick-RNA™ Soil/Fecal Microbe Microprep kit (Zymo Research), we successfully purified approximately 1 μg of total cellular RNA from *E. coli* CFT073 colonized in the mucosal layer of the mouse cecum (Fig 2, lane 1).

In our ongoing analysis of RNA-Seq data from CFT073 grown *in vitro*, the algorithm based approach has facilitated TSS identification, as illustrated in the example of the threonine leader peptide (*thrL*) expression during logarithmic phase growth (Fig 3). The step factor is calculated as the fold increase in the TSS counts (height) in the treated sample relative to the untreated control. For the TEX-enriched RNAs this value equals was ~0.49, whereas the corresponding value in the Cappable-seq enriched RNAs was ~1.61. The average number of reads for the TSS+9 and TSS-9 regions in the untreated control were 72073 and 993. This suggests that there is a 72.6 fold increase in transcription beginning at the TSS without enrichment.

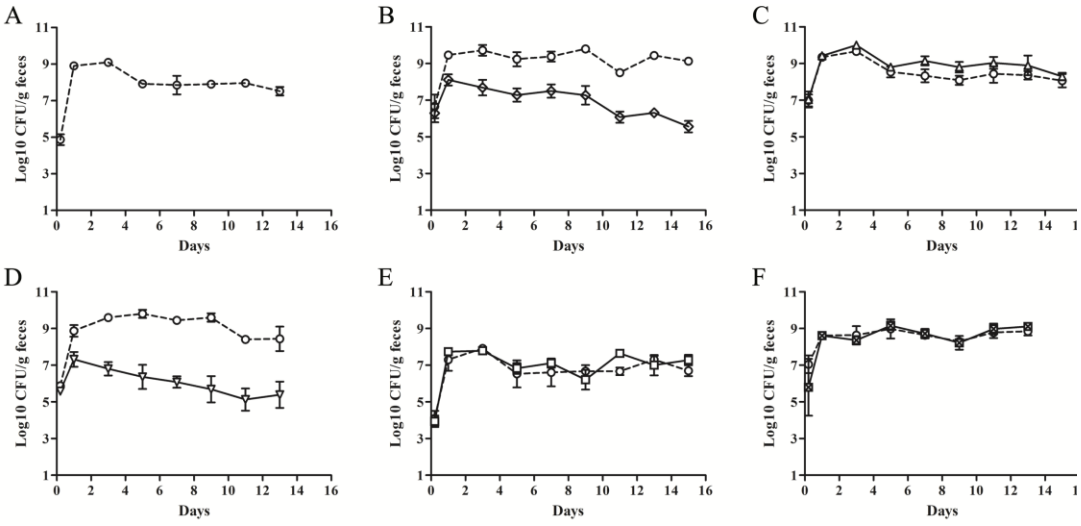

Fig 1 – Colonization and co-colonization of mice with UPEC wild-type and UPEC metabolic mutants. (A) Two sets of 3 mice were fed 10^5 CFU of *E. coli* CFT073 Str^r Nal^r or co-colonized with 10^5 CFU of (B) CFT073 *pykF*, (C) CFT073 *gdhA*, (D) CFT073 *lysP* (E) CFT073 *gnd* or (F) CFT073 *zwf*.

4. Discussion and Conclusion

In this project, we have taken a mechanistic approach to enhancing the current understanding of the physiology of bacteria colonized in the mammalian intestine, focusing on *E. coli* CFT073 (UPEC), the pathogen most frequently cited in urinary tract infections in the United States (Welch et al., 2002). Critical to generating these data was our use of the streptomycin mouse model, which has high experimental reproducibility (Adediran et al., 2014, Leatham et al., 2009, Chang et al., 2004, Jones et al., 2007). To date, very few groups have reported RNA-seq data generated from bacteria stably colonized in the mucosal layer of the gastrointestinal tract (Li et al., 2015, Jenior et al., 2018). As a first step towards performing transcriptomic analysis on bacterial RNA recovered from the mouse cecum, we have developed a protocol which relies on organic extraction in combination with a column based approach to purify total cellular RNA suitable for RNA-seq (Fig 2, lane 1). It is expected that the completed annotation of our *E. coli* CFT073 RNA-seq datasets will provide additional insight on global catabolic pathway utilization during colonization of the cecum.

It has been reported that >75% of recurrent urinary tract infections are caused by the UPEC responsible for the initial infection, suggesting that UPEC such as CFT073 are capable of entering stages of quiescence and giving the pretense that an infection has been cleared (Ejrnaes et al., 2006, Russo et al., 1995). It has recently been shown that *E. coli* CFT073 wild-type grows on glucose M9 minimal media when seeded at $\geq 10^6$ CFU. However, when seeded below this threshold, cells take on a quiescent phenotype that was shown to be disrupted in five *E. coli* CFT073 mini-Tn*5* non-quiescent mutants (Leatham-Jensen et al., 2016). We tested the roles of five genes identified to be involved in a quiescent phenotype observed when CFT073 is seeded at $\leq 10^6$. The CFT073 *pykF* and *lysP* strains showed greater than a 10-fold reduction relative to the CFT073 wild-type when simultaneously colonized at an initial density of 10^5 CFU's in the streptomycin mouse. The absence of a functional pyruvate kinase, which converts phosphoenolpyruvate to pyruvate, reinforces the importance of the glycolytic pathway to colonization of the mouse intestine by commensal as well as pathogenic *E. coli* (Fabich et al., 2008, Chang et al., 2004, Meador et al., 2014, Miranda et al., 2004). However it is unclear why CFT073 *pykF* is non-quiescent *in vitro* (Leatham-Jensen et al.,

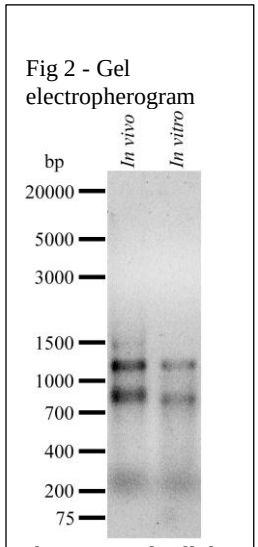

Fig 2 - Gel electropherogram showing total cellular RNA extracted from CFT073 colonized in cecal mucus (lane 1) and RNA from an *in vitro* culture (lane 2).

2016). One hypothesis centers around the accumulation of intracellular phosphoenolpyruvate, a precursor to lysine, methionine, and tyrosine biosynthesis, in CFT073 *pykF* (Siddiquee et al., 2004). Sufficient levels of the three amino acids may stimulate growth in CFT073 *pykF*. Specific transport of lysine in *E. coli* is mediated by the lysine-specific permease system, LysP, but also through the lysine-arginine-ornithine (LAO) system (Steffes et al., 1992). Perhaps the LAO system, which is repressed under nutrient rich conditions *in vitro*, is similarly repressed *in vivo*, relegating the LysP system as the sole mechanism of transporting lysine from the extracellular environment (Rosen, 1971). Previous reports also indicate that *E. coli gdhA* can grow when provided with glucose as the sole carbon source, relying on alternative pathways of glutamate synthesis (Helling, 1994). However, the reason for non-quiescence in CFT073 *gdhA* remains unclear.

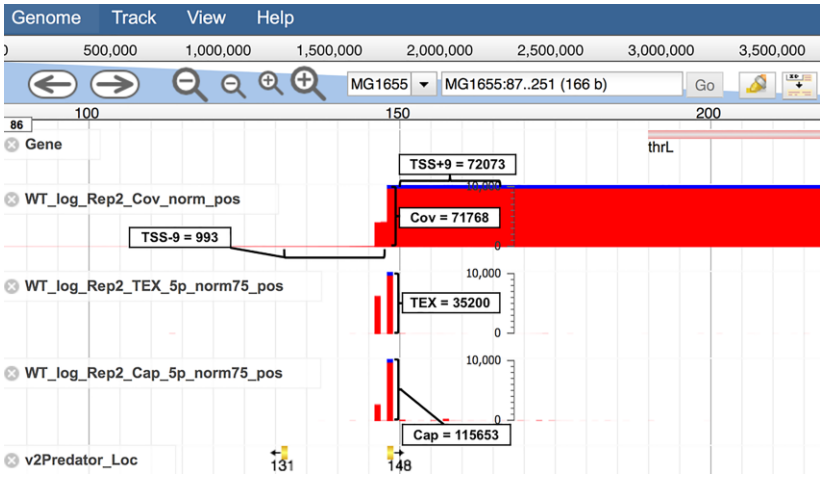

Fig 3 - Annotation of threonine leader peptide (thrL) TSSs in Gene Expression Database. Users view quantitative information (step height, coverage) from multiple replicates simultaneously and annotate transcriptional features with base pair

5. Summary

This report reinforces the importance of targeting total cellular RNA acquired from bacteria colonized in the mucosal layer of the gastrointestinal tract as the most physiologically relevant approach for characterizing nutrient acquisition *in vivo*. Total cellular RNA preparations from bacteria in feces may possibly include RNA's transcripts from transient microorganisms that were ingested but never colonized. We also provide a small glimpse into the complex signaling that controls cellular quiescence, by demonstrating the overlapping importance of two genes, pyruvate kinase and a lysine permease, to both quiescence and being important for colonization. To our knowledge, this is the first report to establish a role for lysine transport in colonization of the streptomycin mouse model. Lastly, we explain three features critical for accurately annotating RNA-seq datasets which remains an ongoing aspect of this project.

6. Appendices

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