Diabetic Complications Consortium

Application Title: "Dissecting biofilm formation and metabolite sensing in diabetic urine"

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1. Project Accomplishments:

The goals of the project were to (1) evaluate the role of diabetic urine metabolites on biofilm formation by uropathogenic *E. coli* (UPEC) and (2) dissect the role of a regulatory network that senses serine and pyruvate changes, in UPEC pathogenesis.

During the award period, we:

- Collected a total of 49 *E. coli* strains from diabetics who presented to VUMC with varying symptoms of UTI. This collection will serve as an invaluable tool, enabling a broad and more representative analysis of the behavior of strains isolated from diabetic individuals with UTI symptoms.
- Collected a total of 251 *E. coli* strains from non-diabetics, who presented with varying symptoms of UTI. These strains will serve as controls for future comparative genomics and metabolomics analyses, comparing how non-diabetic isolates may differ from those isolated from diabetic individuals.
- Determined that in non-diabetic urine, all *E. coli* isolates form variable degrees of biofilm, but biofilm abundance substantially increases in diabetic urine.
- Collected information on the general biofilm phenotypes, such as production of cellulose and adhesive fibers on a qualitative (completed) and quantitative (ongoing) level.
- Established that the metabolite-sensing Two-component systems YpdAB and BtsSR (formerly known as YehUT) play a role in the early stages of infection, when E. coli is sensing a change in metabolic inventory as it enters the bladder.
- Demonstrated that target gene expression in response to increased serine and pyruvate depends on signaling that involves the interaction of the sensor BtsS and the non-cognate partner YpdB.

2. Specific Aims:

Specific Aim 1: **Determine similarities and differences in UPEC biofilms formed in diabetic and non-diabetic urine.**

Results: In order to gain an understanding of how the metabolic inventory in the urine may shape biofilm formation, we first initiated a study in which we banked urine-associated *E. coli* from diabetic and non-diabetic individuals, under IRB #151465. A total of 300 urine-associated *E. coli* strains were collected over a one-month period (**Figure 1**), of which 49 came from diabetic patients (**Figure 1-2**).

Among some critical observations, pointing towards the higher levels of bacteriuria in the diabetic population, was the fact that 44% of diabetic patients asymptomatically carried *E. coli*, compared to 23% of non-diabetic individuals (Figures 2-3). In addition, of the three urosepsis isolates collected in the study, two came from diabetic individuals (**Figure 2-3**) and isolate VUTI159 exhibited the highest biofilm formation during growth in laboratory conditions (**Figure 2**).

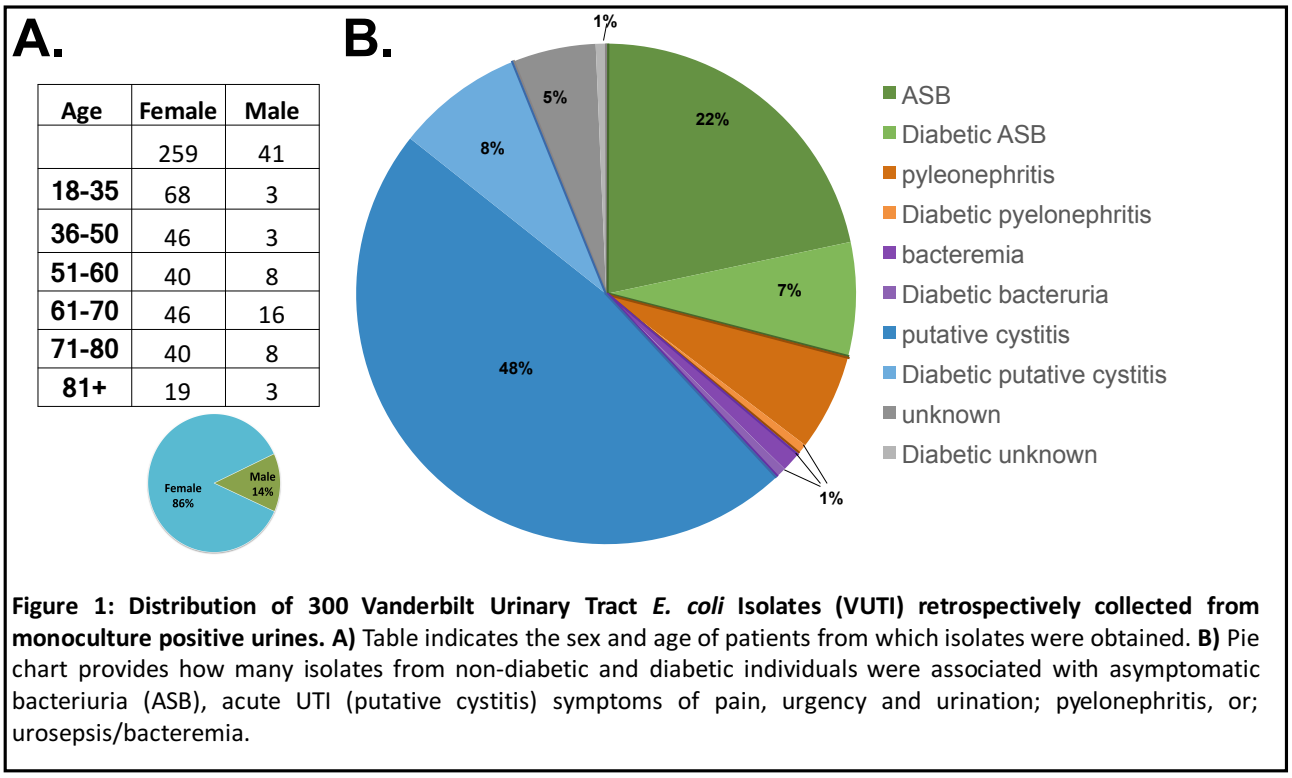


Figure 1: Distribution of 300 Vanderbilt Urinary Tract *E. coli* Isolates (VUTI) retrospectively collected from monoculture positive urines. A) Table indicates the sex and age of patients from which isolates were obtained. **B)** Pie chart provides how many isolates from non-diabetic and diabetic individuals were associated with asymptomatic bacteriuria (ASB), acute UTI (putative cystitis) symptoms of pain, urgency and urination; pyelonephritis, or; urosepsis/bacteremia.

Initially, we evaluated the ability of all collected isolates to form biofilm in laboratory growth conditions. Colorimetric measurement of biomass demonstrated that all diabetic isolates could form biofilms under both conditions, albeit at varying degrees and that gross biofilm abundance did not significantly differ from the biofilms formed by *E. coli* isolated from non-diabetic individuals (**Figures 2-3** and [1]).

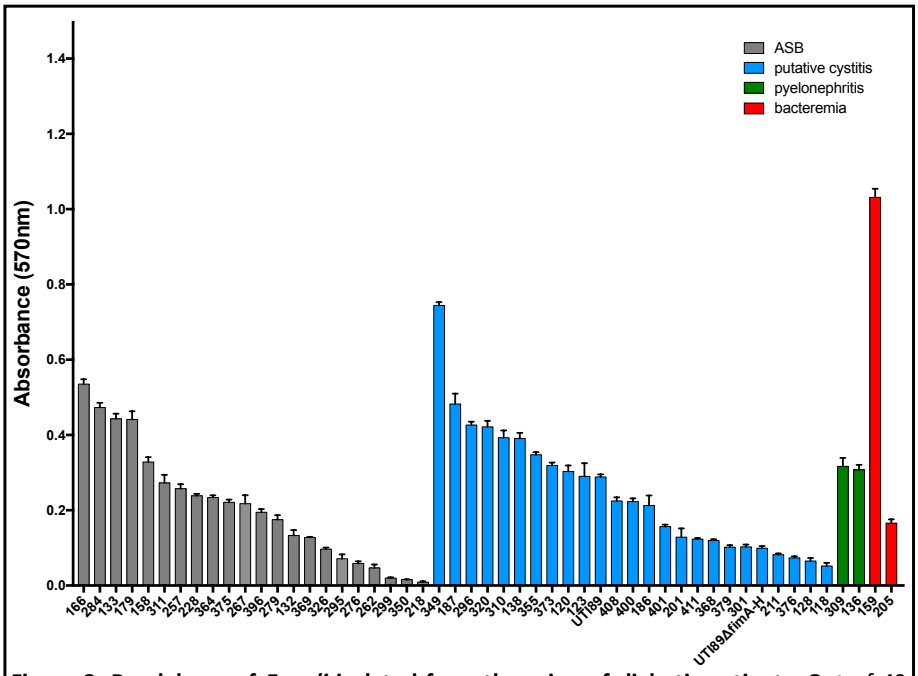


Figure 2: Breakdown of *E. coli* isolated from the urine of diabetic patients. Out of 49 collected strains, 22 were from asymptomatic bacteriuria (ASB), 25 came from patients who presented with acute UTI (putative cystitis) symptoms of pain, urgency and urination; 2 were isolated from patients with pyelonephritis, and; 2 were isolated from patients who had urosepsis. The graph indicates biofilm abundance during growth in laboratory conditions, measured using a colorimetric, crystal violet staining assay.

We then asked whether biofilm formation in diabetic urine would be enhanced in all strains.

Our initial hypothesis was

that biofilm formation would be different during growth in diabetic urine, due to the different metabolic inventory available to E. coli. If this hypothesis is true, then all isolates, regardless of whether they were isolated from diabetic or non-diabetic patients would exhibit increased biofilm formation.

Urine from otherwise healthy diabetic individuals under IRB # 161085 was collected for these studies. At each day of collection, glucose levels in the urine were checked at the clinic; the urine samples were de-identified, pooled together and provided to members of the Hadjifrangiskou lab. Collected urine was filter-sterilized through a 22micrometer filter to remove any potential microbial contamination and was used immediately to set up biofilm assays. Controls for the assays always included a "blank", in which collected urine was incubated without the addition of *E. coli* to ensure that no growth would result that is attributed to contamination from the urine. In parallel studies, the same *E. coli* strains were seeded in pooled urine from healthy donors without diabetes. We first selected a representative group of isolates from patients with different symptoms, ranging from asymptomatic bacteriuria (ASB), to cystitis, pyelonephritis and bacteremia. In all analysis, the non-diabetic, well-characterized isolate UTI89 was included as a control. The results obtained indicated that when grown in diabetic urine, there was a significant increase in biofilm formation by the majority of strains tested (**Figure 4**), suggesting that our hypothesis is true and that metabolic inventory in

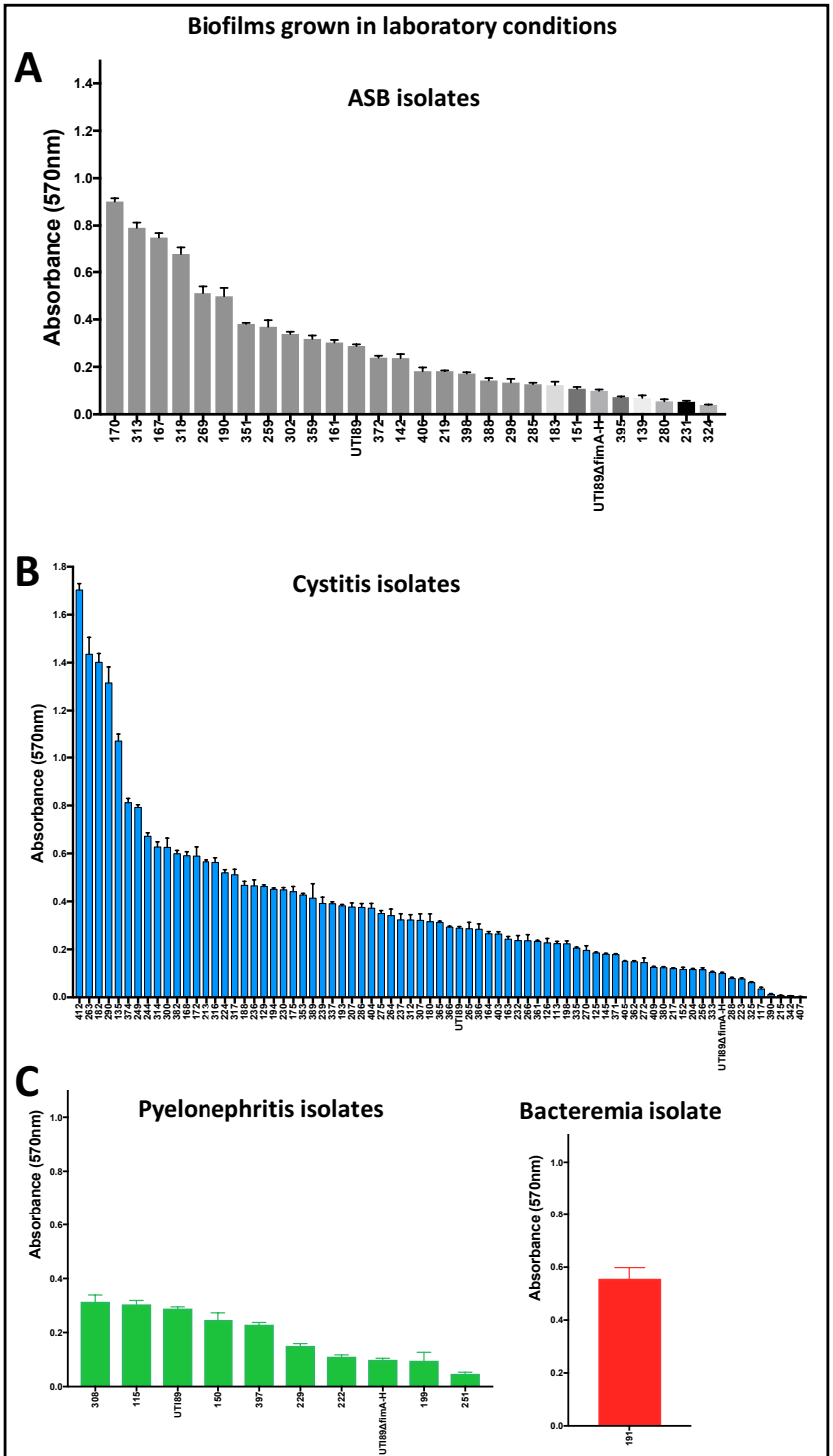


Figure 3: Biofilm trends of isolates from non-diabetic patients with no other complications during growth in laboratory conditions. Out of 251 collected strains, 108 were isolated from non-diabetic individuals with no other complications such as catheters, pregnancy or spinal cord injury. Of the 108 uncomplicated cases, 25 were from asymptomatic bacteriuria (ASB), 74 came from patients who presented with acute UTI (putative cystitis) symptoms of pain, urgency and urination; 8 were isolated from patients with pyelonephritis, and; 1 was isolated from patients who had urosepsis. The graphs indicate biofilm abundance during growth in laboratory conditions, measured using a colorimetric, crystal violet staining assay.

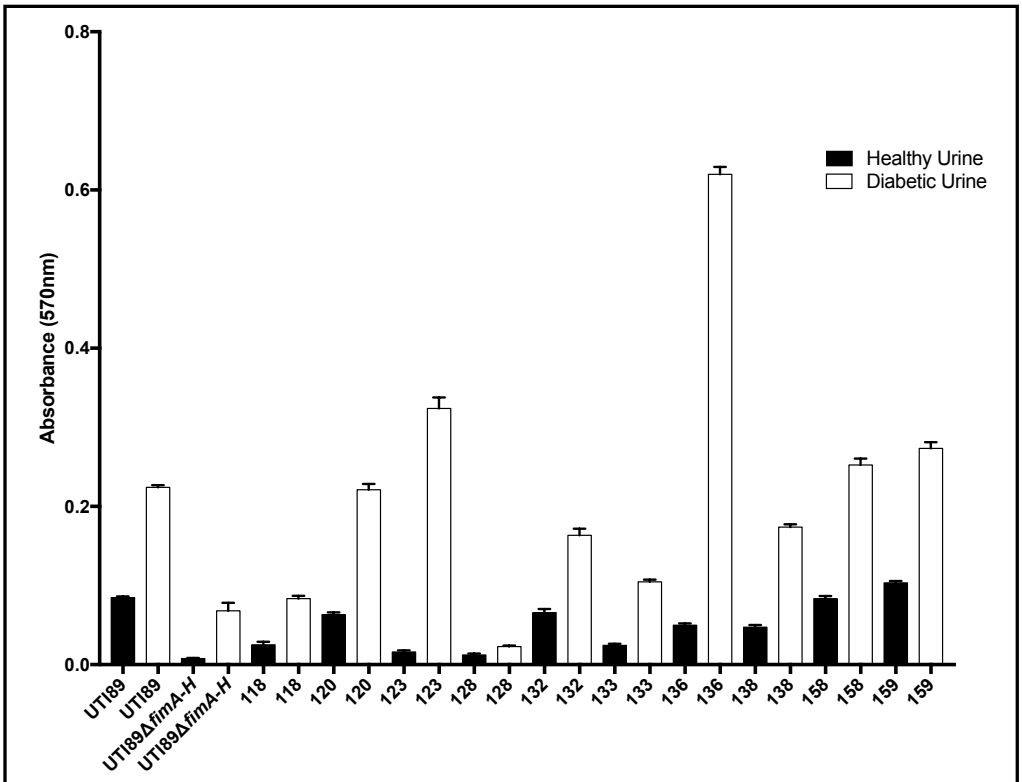
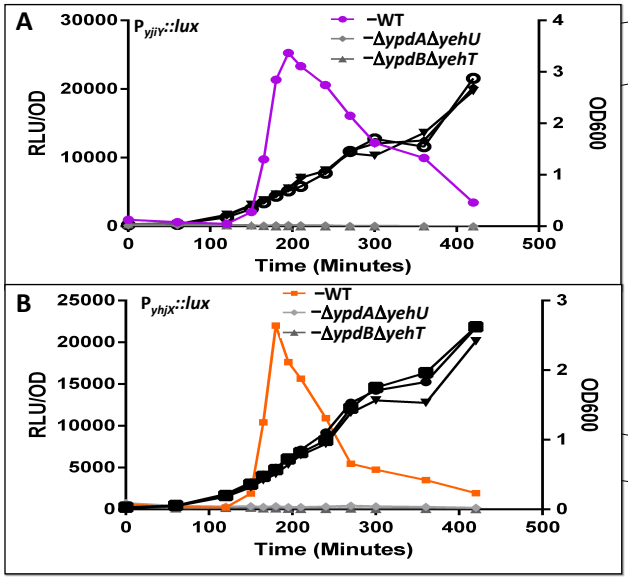


Figure 4: Diabetic urine enhances biofilm formation in all strains tested. The graph indicates biofilm abundance during growth in urine from healthy donors (black bars), versus growth in urine collected from diabetic individuals (white bars). Biofilm abundance was measured using a colorimetric, crystal violet staining assay. An average of two biological replicates with two different batches of pooled diabetic urine is shown.

diabetic urine overall enhances biofilm formation, regardless of whether the isolates are from diabetic or non-diabetic patients.

Having observed this result, we then embarked on analyzing biofilm composition and proteomic profiles. This is an ongoing study, the final results of which will be used as preliminary data for the submission of an R01 application seeking to continue this study.

Specific Aim 2: Define the role of YpdA and YehU bacterial sensors in enhancing diabetic UTI.



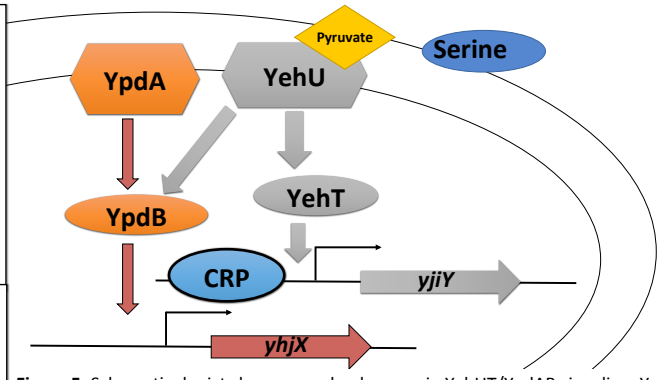


Figure 5: Schematic depicts knowns and unknowns in YehUT/YpdAB signaling. YpdA and YehU are membrane-embedded histidine kinases. YehU has been shown to bind and respond to pyruvate. Both YpdA/YpdB and YehU/YehT respond to changes in serine abundance. YpdB and YehT are the corresponding response regulators for YpdA and YehT respectively. The only currently known targets for YehU/YehT and YpdA/YpdB are genes *yhjX* and *yjiY* that encode putative membrane-embedded transporters.

Graphs depict the transcriptional activity of the *yjiY* **(A)** and the y*hjX* **(B)** promoters over time during growth of *E. coli* in lysogeny broth (LB). Promoter activity is measured as luminescence (in RLU/OD600) over time, using transcriptional fusions of either the *yjiY* or the *yhjX* promoter to the *lux* operon. The right y-axis indicates the corresponding culture optical density for each strain (depicted in the black lines) at 600nm (OD600) (squares: WT; circles: Δ*ypdA*Δ*yehU*; inverted triangles: Δ*ypdB*Δ*yehT*). Graphs shown are representative of at least 9 biological experiments.

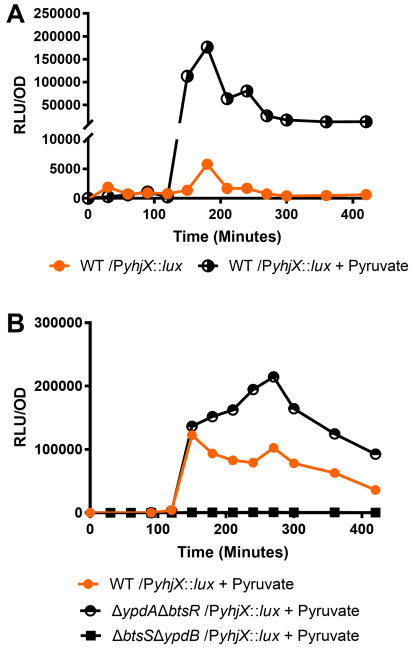


Figure 6: Graphs depict the transcriptional activity of the *yhjX* promoter in the presence of pyruvate in (A) wild-type (WT) UPEC and (B) in mutants lacking non-cognate partner components of YpdAB and BtsSR. Panel in (B) indicates that loss of sensor BtsS and non-cognate partner YpdB leads to complete loss of yhjX target gene upregulation. This suggests that target gene expression in response to the pyruvate metabolite depends on cognate partner interactions between BtsS and YpdB. The graphs shown are representative of 3 independent experiments.

Results: Two-component systems (TCSs) dictate many bacterial responses to environmental change via the activation of a membrane-embedded sensor kinase, which has molecular specificity for a cognate response regulator protein. However, although the majority of TCSs operate through seemingly strict cognate protein-protein interactions, there have been several reports of TCSs that violate this classical model of signal transduction. Our group has recently demonstrated that some of these cross-interacting TCSs function in a manner that imparts a fitness advantage to bacterial pathogens. One set of TCSs we proposed to investigate in aim 2 of the awarded funding application, are the metabolite-sensing TCSs YpdA/YpdB and YehU/YehT (which has since been renamed to BtsS/BtsR (**Figure 5** and [2]). In previous studies we demonstrated that the YpdA/YpdB and BtsS/BtsR TCSs are active during acute and chronic UTI in a murine model of infection, as evidenced by the upregulation of their target genes *yhjX* and *yjiY* [2, 3]. We were interested in dissecting the role of these systems in UPEC pathogenesis, because they sense two metabolites that are found in higher abundance in the urine of diabetics: serine and pyruvate (**Figures 6A and 7**). Previous studies by our lab and others indicated that UPEC utilize amino acids as opposed to glucose during infection [4, 5]. Here, we show that target gene upregulation in response to the sensing of pyruvate depends on non-cognate interactions between the sensor BtsS and the response regulator YpdB (**Figure 6B**). We also demonstrate that deletion of genes that are involved in serine import and utilization lead to abrogation of signaling by BtsSR and YpdAB, (**Figure 7**) indicating a role for these signaling systems in nutrient acquisition by

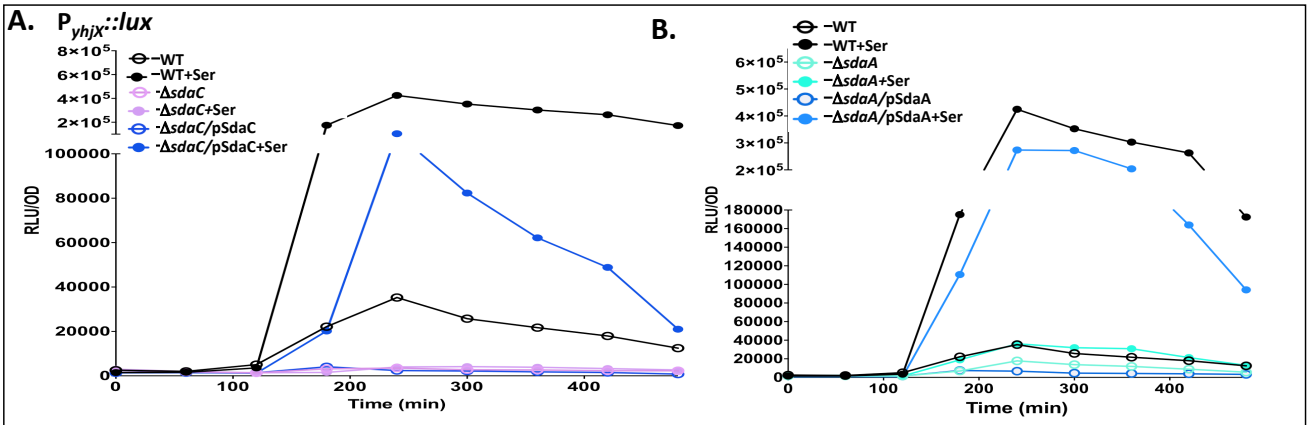


Figure 7: Graphs depict the transcriptional activity of the *yhjX* promoters driving the expression of the *lux* operon over time during growth of *E. coli* in LB, in the presence and absence of 1mM serine. Serine was added to each culture at 120 minutes of growth. The graphs shown depict the geometric mean of 3 independent experiments. Panel A indicates the effects of deleting the gene for the main serine transporter SdaC on target gene expression. Panel B indicates the effects of deleting the gene for serine-pyruvate inter-conversion enzyme SdaA on target gene expression. Complementation constructs "pSdaC" and "pSdaA" were created in the pBAD33 plasmid backbone, under the control of their own promoter.

UPEC during infection. To test this we first tested the fitness of UPEC mutants deleted for *btsSR* or *ypdAB* in an acute UTI model (**Figure 8**). We observed a reduction in early colonization for both mutants (**Figure 8A**), suggesting that metabolite sensing via these two systems is critical for bladder infection. Moreover, deletion of the *btsSR* system led the bacteria to have a very subtle, but reproducible tropism for the kidney (**Figure 8B**). We are now in the process of investigating the effects of mutants deleted for BtsS and YpdB, given that target gene upregulation depends on non-cognate interactions between these two proteins. We are also in the process of investigating how deletion of the BtsS/YpdB influences diabetic UTI progression.

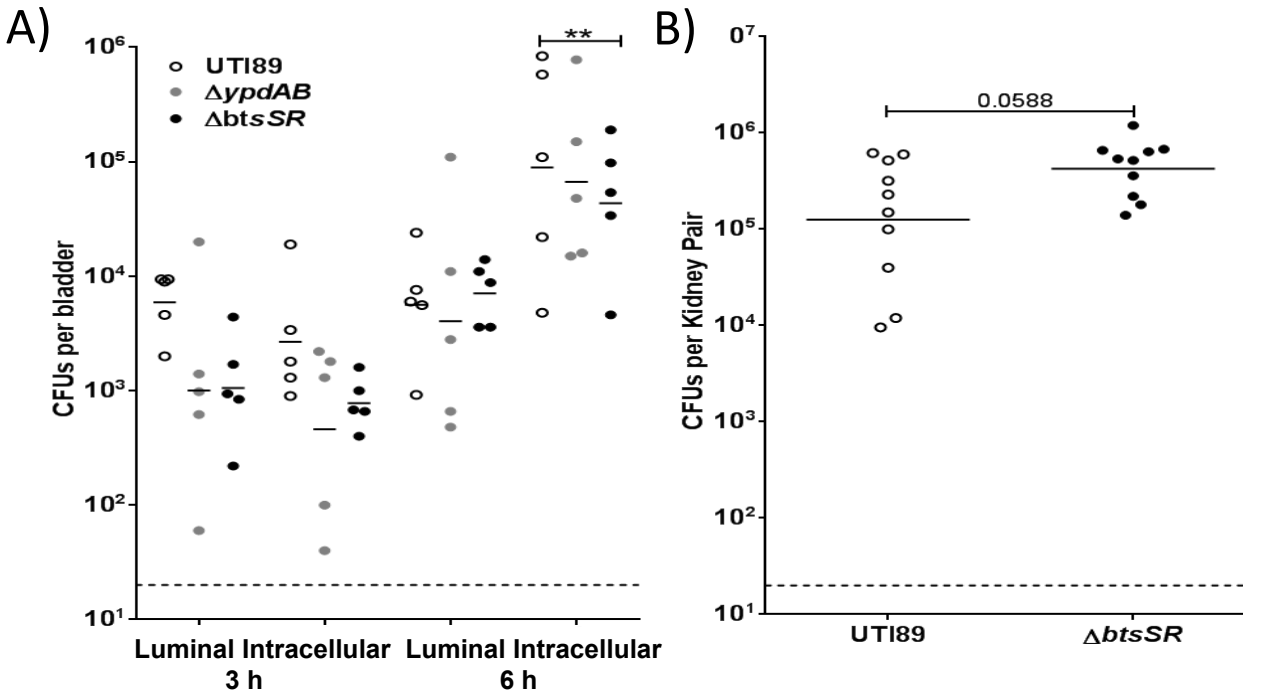


Figure 8: Deletion of *ypdAB* or *btsSR* decreases UPEC fitness in acute UTI. Bacterial burdens within the murine bladder **(A)** and kidneys **(B)** during acute UTI. **A)** Bladders were washed in PBS to remove non-adherent bacteria (luminal). Intracellular bacteria were quantified after treating bladders for 2 h with gentamycin, homogenized, and enumerated. Statistical analysis was performed using 2-way ANOVA with Dunnet's multiple comparison test. P-values: **, *P*=0.0052. **B)** Kidneys were homogenized and enumerated for CFU. Statistical analysis was performed using Student's t-test. P-values:: Shown above comparison bars.

3. Publications stemming from work funded from this award:

Eberly, A.R., et al., *Biofilm Formation by Uropathogenic Escherichia coli Is Favored under Oxygen Conditions That Mimic the Bladder Environment.* Int J Mol Sci, 2017. **18**(10).

Steiner B.D. et al., *Evidence of Cross-Regulation in Two Closely Related Pyruvate-Sensing Systems in Uropathogenic Escherichia coli* Journal of Membrane Biology, In press.

4. References Cited

1. Eberly, A.R., et al., *Biofilm Formation by Uropathogenic Escherichia coli Is Favored under Oxygen Conditions That Mimic the Bladder Environment.* Int J Mol Sci, 2017. **18**(10).
2. Behr, S., et al., *Identification of a High-Affinity Pyruvate Receptor in Escherichia coli.* Sci Rep, 2017. **7**(1): p. 1388.
3. Conover, M.S., et al., *Metabolic Requirements of Escherichia coli in Intracellular Bacterial Communities during Urinary Tract Infection Pathogenesis.* MBio, 2016. **7**(2): p. e00104-16.
4. Alteri, C.J., S.N. Smith, and H.L. Mobley, *Fitness of Escherichia coli during urinary tract infection requires gluconeogenesis and the TCA cycle.* PLoS Pathog, 2009. **5**(5): p. e1000448.
5. Hadjifrangiskou, M., et al., *A central metabolic circuit controlled by QseC in pathogenic Escherichia coli.* Mol Microbiol, 2011. **80**(6): p. 1516-29.