YpdAB and BtsRS: Interconnected Signaling Networks with Pyruvate and Acid Sensing Functions in Uropathogenic Escherichia coli

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Dedicated in memory of Jessie Mae Jones Sapp who taught me to always ask questions.

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Introduction

Signal Transduction Systems: The Big Picture

Biology can be succinctly described as the study of living systems. However, despite this distinction between living and non-living systems biological organisms must still operate under the laws of physics and chemistry. Understanding the chemical and physical basis of biological processes, and how organisms interpret the chemical and physical information around them, has been an area of scientific interest for decades. The level of complexity inherent in biological systems requires that organisms maintain precise control over their physiological makeup. Control of cellular physiology relies on an organism being able to simultaneously sense and respond to a dizzying array of information regarding their intracellular homeostasis as well as the environmental conditions, threats, and resources around them. Such sensory information comes in the form of molecular inputs, and over the course of evolution, organisms have developed exquisite information processing systems that convert biochemical data into functional outputs via intracellular signaling pathways (refer to Burgoyne & Petersen's excellent *Landmarks in Intracellular Signaling*, 1997, for a compilation of several key and historic signaling papers). The systems that handle this conversion of biochemical information into functional outputs can be collectively referred to as cellular signaling systems and it is not an exaggeration to state that understanding the mechanistic and kinetic details of these systems is fundamental to understanding biology more broadly. After all, in some manner or another biochemical signaling events influence or dictate every complex biological activity. Specifically, the process of a molecular signal being transmitted from a cell's exterior into the cell is referred to as a signal transduction event and the systems that allow for and manage this inward flow of information are termed signal transduction systems.

The field of signaling research is both large and rapidly growing. As one might expect, throughout the course of evolution signal transduction systems have evolved to come in many different forms and can function through varied and widely divergent mechanisms of action. However, equally as impressive is the staggering diversity of function that can be observed within a single class of signal transduction systems. Take as an example the G-Protein Coupled Receptors (GPCRs), which are found in many eukaryotes from fungi to animals. GPCRs are embedded in the cellular membrane via seven transmembrane domains and possess an extracellular domain that can interact and bind with molecules on the exterior of the cell, as well as an intracellular domain, which is associated with proteins known as G-proteins. Upon ligand binding, GPCRs undergo a conformational change, which facilitates interaction with and activation of their associated G-protein; followed by the downstream physiological effects associated with that G-protein's activation (Kobilka, 2007; Strader et al., 1994). One thing that makes signaling systems such as GPCRs so interesting is that this relatively simple sounding mechanism of action can be modified and altered to perform an incredible array of functions within an organism. There are different types of G-proteins that can have cooperative or antagonistic effects on cellular physiology. GPCRs are oligomeric and exist in various homo- or hetero-dimer conformations, which imparts differences in sensitivity to and preference towards various ligands and G-protein partners. GPCRs possess a wide diversity of ligand binding domains which allows them to respond to many different biochemical cues. Additionally, some GPCRs possess the ability to activate multiple G-proteins or to be selectively biased towards particular G-proteins in different tissues, allowing GPCRs to not only modulate many aspects of cellular physiology but to actually help differentiate distinct cell populations within an organism. Furthermore, cells have the capacity to differentially turn these GPCRs and G-proteins on or off to maintain fine control over cellular physiology and signaling. (Deupi & Kobilka, 2007; Katritch, Cherezov & Stevens, 2013; Kobilka & Deupi, 2007; Lefkowitz, 2013; Offermanns, 2003). GPCRs are thus able to regulate an impressively diverse range of biological activities and cellular

processes all from the same "basic" blueprint of the GPCR (Wettschureck & Offermanns, 2005). An organism that can tightly regulate its cellular physiology in a wide variety of conditions has a distinct fitness advantage over less efficient and less precise organisms. As such, evolutionary selective pressures have produced tremendous diversity both within and across signal transduction system classes in order to maintain fitness in the face of many cellular and organismal needs.

A fantastic example of a signal transduction system that has evolved to fulfill a highly specific purpose is the toll-like receptor family (TLRs). TLRs have evolved to recognize structurally conserved molecules of microbial origin in order to initiate an immune response geared at fighting off potential pathogens. There are many different members in the TLR family and they are capable of binding and "recognizing" microbial molecules such as flagellin proteins, lipoteichoic acid moieties (a component of gram-positive bacterial cell walls), lipopolysaccharide (aka LPS, a component of gram-negative bacterial cell walls), and much more (Akira & Takeda, 2004; Kawasaki & Kawai, 2014). However, even a class of signaling systems as seemingly straightforward in purpose as TLRs can generate many interesting questions. For example, what sort of interplay is there between the beneficial bacteria of the human microbiome and TLR expressing cells? What evolutionary back and forth has allowed for the body to harbor trillions of "good" bacteria while at the same time incessantly attempting to find and destroy "bad" bacteria? In fact, there is growing evidence that commensal bacteria in humans do not simply signal their benign or beneficial nature to human immune cells but that they actually interact extensively with the immune system, in a sense "training" immune cells, and that this signaling can influence disease states such as systemic inflammation (Valentini et al., 2014; Yiu, Dorweiler & Woo, 2017). Thus, even a class of signaling systems whose function was previously thought to be well understood may actually be playing important roles in processes as complex and important as the development of the immune system. Clearly, if we as scientists wish to better understand the puzzles of biology, it is prudent for us to continue studying and investigating cellular signaling

systems. In particular, I believe that signaling systems involved in communication between or across domains of life, such those at the interface of bacteria and humans, will be a fruitful area of future research and will have much to tell us about human evolution and health.

It can be daunting to orient oneself in a field as large and complex as cellular signaling. Not only is there decade's worth of literature and established knowledge to familiarize oneself with, the very nature of cellular signaling systems can make them difficult to study in a well-controlled but biologically relevant experimental context. Within multicellular organisms, such as humans, there are many cell populations that are highly differentiated and that may possess only the signal transduction machineries needed for their particular locale or function within the greater organism. Many of these cellular signaling systems have evolved to fulfill highly specific functions *in vivo* and it can be challenging to manipulate these systems and understand them properly in the laboratory. Studying signaling networks in single-celled lifeforms, such as bacteria, allows us to circumvent many of these issues. Bacteria such as *E. coli* are genetically tractable and have a plethora of genetic tools available for easy manipulation and study of proteins. Bacteria can be grown quickly and easily in a laboratory and with the use of murine infection models and other tools it is possible to accurately analyze bacterial behavior and activity *in vivo*. Moreover, focusing on single-celled organisms provides us with an opportunity to study a single cell that must respond to the entirety of its environmental inputs alone and that must be able to access and utilize the entirety of its genome at a moment's notice. Given that any one bacterial cell must be able to perform all of the functions of life, studying bacterial signaling systems can give us incredible insight into the biochemical processes upon which life is predicated. Additionally, bacteria can have a tremendous impact on human health, both as symbiotic members of the host microbiome or as pathogens that can infect and cause harm. Studying bacterial signaling networks allows us to better understand how these bacteria live, reproduce, and interact with their human host and one another.

Signal Transduction Systems: The Biomedical Perspective

Despite their variety in form, function, and distribution it is worth emphasizing again that all signal transduction systems exist to perform the same fundamental function: translating an external cue into a cellular response. This *raison d'être* of signaling systems is worthy of reiteration because it is why signaling systems have become fantastic drug targets in the modern age. Pharmacology operates on the assumption that we can alter cellular and systemic physiology and biochemistry to the benefit of human health. With signaling systems playing such a fundamental role in altering the physiology of an organism it is easy to see why better understanding signaling networks can allow us to better understand human health and how to augment it. In a related fashion, it becomes clear that understanding signaling systems and physiology in organisms such as pathogenic bacteria may allow us to design new therapeutics that thwart pathogens without detrimentally impacting human health. Bacteria, with their staggering diversity of form and habitat, have evolved a fantastic array of signal transduction systems allowing them to perform the many functions of life. It is therefore highly plausible that pharmaceutical interventions aimed at disrupting these signal transduction networks might be able to reduce bacterial fitness or reprogram bacterial niche preference.

Of particular interest to the biomedical research field are the signal transduction networks essential for pathogenic bacteria to successfully survive in and infect a host. Myriad sensory inputs must be synthesized and cohesively processed so that a pathogen can gain entry into the host and subsequently navigate to its appropriate niche(s) within that host. Throughout a pathogen's lifecycle it must either scavenge or compete with the host for scare nutrients (Prentice, Ghattas & Cox, 2007; Skaar, 2010), it must be able to replicate in order to pass on its genetic information, and it may need to repair itself in diverse environments that have very different resources available. A pathogen may need to become motile in order to move or to flee and pathogens need be able recognize surfaces and cells that they can attach to or invade (Chaban, Hughes & Beeby,

2015; Ribet & Cossart, 2015; Spaulding & Hultgren, 2016). Some pathogens may form biofilms within the host, creating protected bacterial communities that are insulated from antibiotics and immune cells, and then upon receiving the appropriate cue may disperse from those biofilms at a later time (Flemming et al., 2016; Hadjifrangiskou et al., 2012; Høiby et al., 2010; Roilides et al., 2015). Additionally, nearly all pathogens in some form or another seek to evade, thwart, or compromise the immune system (Finlay & McFadden, 2006; Reddick & Alto, 2014). It is remarkable that bacterial pathogens as small as a half micro-meter in diameter are capable of performing all of the aforementioned tasks. Fundamentally, it is bacterial signal transduction systems that control all of these various activities, and by disrupting these signaling networks we may be able to disrupt bacterial pathogenesis (Freeman, Dorus & Waterfield, 2013; Hadjifrangiskou et al., 2011; Kostakioti et al., 2009; Skerker et al., 2005; Tipton & Rather, 2016; Tiwari et al., 2017; Tobe, 2008).

Bacterial Two-Component Systems: Mechanisms and Form

A variety of signal transduction machineries have evolved in bacteria to handle the tasks of life and chief amongst them are the two-component systems (TCSs). In their most basic form TCSs are composed of two cognate proteins: a membrane-embedded sensor and a cytosolic response regulator. The membrane-embedded sensor is a histidine kinase that auto-phosphorylates at a conserved histidine residue and upon phosphorylation can subsequently activate the cytosolic protein via phosphotransfer to a conserved aspartate residue (Hoch, 2000). This basic mechanism of action drives TCSs that are essential for bacterial cell wall metabolism (and as a consequence growth and division of bacteria) (Okada et al., 2010; Skerker et al., 2005) , bacterial sensing and acquisition of nutrients (Cai et al., 2013), the expression of virulence factors (Tobe, 2008), and control of bacterial cellular defenses in response to hazardous environmental stress, pH, and osmolarity (Gebhardt & Shuman, 2017; Tipton & Rather, 2016). TCSs have even

been directly implicated in numerous forms of antibiotic resistance (Gebhardt & Shuman, 2017; Guckes et al., 2017; Kellogg et al., 2017; Macfarlane, Kwasnicka & Hancock, 2000).

Sensor histidine kinases exist as dimers in the membrane and are composed of two primary domains: the input domain which receives or responds to an extracellular cue and the intracellular transmitter domain which upon activation auto-phosphorylates and is responsible for phosphotransfer to the response regulator protein. The response regulator protein is typically composed of two domains: a receiver domain and an output domain. The receiver domain of the response regulator is so named because this region of the protein contains the conserved aspartate which is phosphorylated during signal transduction (Bhate et al., 2015; Casino, Rubio & Marina, 2010; Gao & Stock, 2009; Robinson, Buckler & Stock, 2000; Zschiedrich, Keidel & Szurmant, 2016). A basic schematic depicting a TCS signal transduction cascade can be seen in **Figure 1**. It is worth noting here that the amino acids surrounding the sensor's conserved histidine residue and the regulator's conserved aspartate residue form the primary basis of biochemical specificity between TCS partners (Podgornaia & Laub, 2013). After phosphorylation, the response regulator will undergo a conformational change freeing the output domain to interact with its cellular targets. Response regulatory proteins are most often transcriptional regulators that bind DNA although they may also bind RNAs, interact with proteins, or modulate other signaling networks (Galperin, 2010). Response regulator proteins and their cognate histidine kinases are frequently transcribed as part of a genetic operon. Operons are a genomic organizational strategy frequently found in bacteria that allow for one promoter to control the simultaneous transcription of a cluster of genes arranged one after another on the chromosome, as depicted in **Figure 2**.

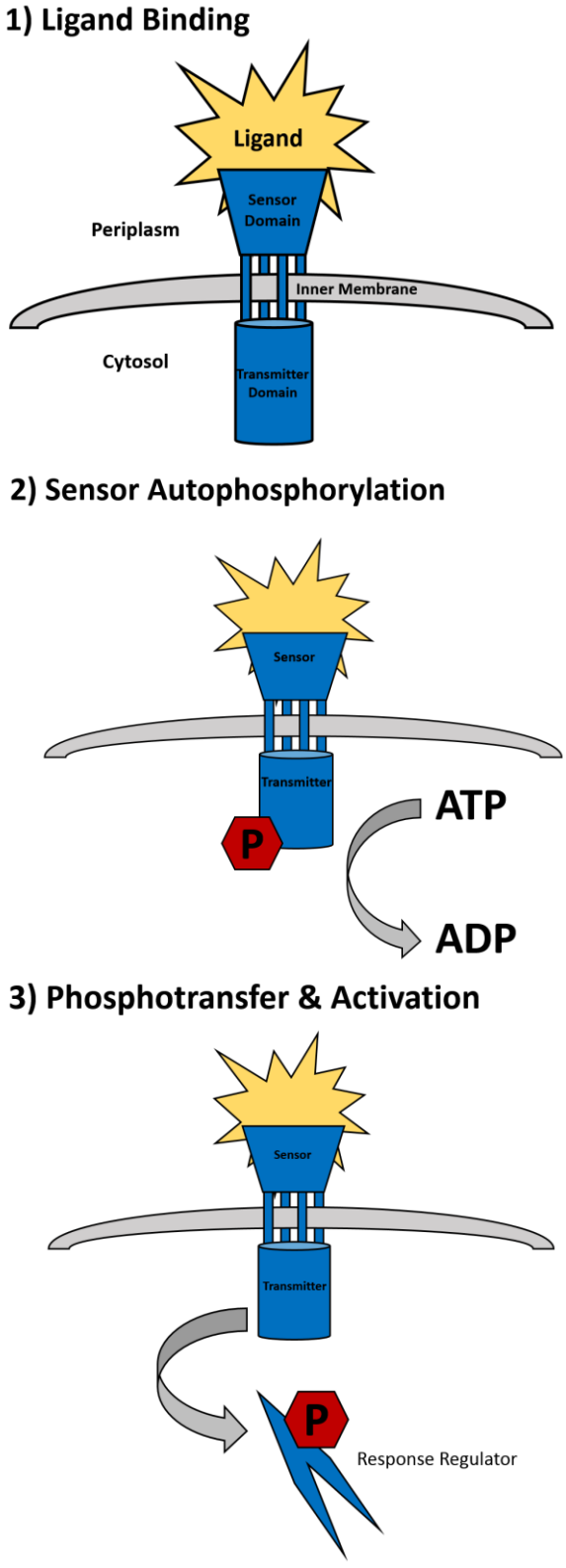


Figure 1. Basic schematic of TCS signal transduction

This schematic depicts the sequential order of events that occur during signal transduction in a prototypical TCS.

1) A ligand binds with and activates the extracellular sensory domain of the sensor histidine kinase.

2) The sensor histidine kinase utilizes ATP to autophosphorylate a conserved histidine residue and undergoes a conformational change preparing it to interact with its cognate response regulator protein.

3) The sensor histidine kinase catalyzes the transfer of the aforementioned phosphate to a conserved aspartate residue on the response regulator protein, activating the response regulator and allowing it to perform its functions within the cell.

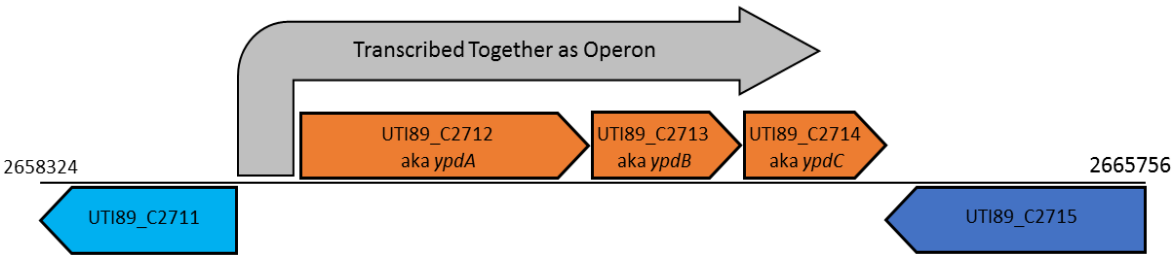


Figure 2: Schematic of the *ypdABC* operon in UTI89

This schematic depicts the UTI89 genome from position 2658324 to 2665756. As can be seen, bacterial genes are frequently organized into clusters called "operons" under the control of a single upstream promoter site, which allows for efficient up and down-regulation of systems or sets of related genes.

This genetic organization allows for efficient up- or downregulation of TCSs in response to the constantly fluctuating needs of the bacterium and provides for integrated genomic control over a given TCS (Groisman, 2016; Mitrophanov & Groisman, 2008). In terms of cellular organization, the sensor and regulator proteins are found in close proximity facilitating rapid interaction in response to signals and minimizing interference from proteins not involved in the signal transduction cascade (García Véscovi, Sciara & Castelli, 2010). While traditionally, TCSs were thought to not engage in beneficial cross-interaction with other TCSs, research in the field is identifying systems in which non-cognate sensor and regulator interactions are needed for proper signal transduction (Guckes et al., 2017; Guckes et al., 2013; Steiner et al., 2018; Wei et al., 2017). The work described in this thesis explores one such example of cross-regulation between TCSs.

Two-Component Systems: Evolution and Function

Although TCS signal transduction cascades were traditionally thought to be highly insulated recent research has begun to elucidate instances of beneficial cross-talk and interconnectivity between systems. TCSs are thought to have a shared evolutionary basis as evolutionary analyses have suggested that they typically arise via gene duplication events (Salazar & Laub, 2015). Following a gene duplication event two identical TCSs will exist for a period time. Eventually, the acquisition of mutations leads to divergence and to differences in signal specificity, response output, and sensor-regulator affinity. Divergence allows the two TCSs to be maintained in the face of selective pressures acting to minimize the bacterial genome size and reduce unbeneficial crosstalk between signaling systems (Mira, Ochman & Moran, 2001; Rowland & Deeds, 2014). TCSs are by their nature mosaic proteins, meaning that they are composed of multiple functional domains. Mosaic proteins are frequently hotspots for evolutionary activity because the modular nature of these proteins allows for domains to be swapped amongst one another in an process referred to as domain shuffling; an evolutionary event in which genetic

information encoding for protein domains become swapped or shuffled with the genes encoding for different proteins (Di Roberto & Peisajovich, 2014). If the domains of various protein classes are similar enough to create functional proteins then domain shuffling can result in the creation of fusion proteins that combine the unique characteristics of the domains comprising them (Di Roberto & Peisajovich, 2014). In the context of bacterial signaling systems this means that TCSs can be created via domain shuffling that contain sensory input domains from other types of bacterial proteins such as chemotaxis proteins or threonine kinases and that TCSs can gain response regulator output domains that allow them to interact with RNAs, proteins, or gene loci they did not previously interact with. The process of acquiring sensory or output domains from other classes of signaling proteins, as well as the more basic shuffling of domains between TCSs, is a powerful mechanism for creating evolutionary diversity within bacterial signaling networks (Alm, Huang & Arkin, 2006; Pao & Saier, 1995). Evolutionary adaptations can also be driven by a variety of single point mutations within the genes encoding for TCS. Sensor kinase genes may accumulate point mutations that alter protein regions within the sensor-regulator interaction interface or that are involved in ligand binding and signal differentiation. Response regulator genes are also subject to point mutation driven evolution. Single point mutations within response regulator genes can lead to alterations in the sensor-regulator interaction surface which can change the affinity between that regulator and its cognate sensor. Mutations within response regulator genes can also produce regulator proteins that vary in the manner, location, and frequency with which they interact with their associated promoter regions or RNA polymerases; such mutations can have a substantial impact on bacterial genomic regulation. (Capra & Laub, 2012; Koretke et al., 2000; Salazar & Laub, 2015)

Newly arisen TCSs are subject to heavy selective pressure to differentiate them from their ancestral progenitor and tend to quickly acquire mutations involved in stimulus sensing and stimulus response. As such, the TCSs that are retained are typically those that rapidly acquire alterations in the sensory processing domains, the output domains, and/or at the interface

between the kinase and regulator catalytic domains. Once mutations begin to differentiate stimuli or stimuli responses between the new and ancestral TCS the interface between sensor and regulator proteins becomes an area of particularly important mutational acquisition as this is where specificity between cognate partners is coded. Computational analyses have revealed that there is extensive coevolution of amino acids at the molecular interface between kinase sensors and response regulators. This co-evolution is a fine balancing act that requires the initial acquisition of neutral or nearly neutral mutations followed by more disruptive alternating mutations. The sequential evolution between the two proteins allows the domains to continue to interact and communicate whilst they are differentiating from their ancestral progenitors. Over time, this alternating acquisition of mutations can lead to a fully differentiated TCS that does not interact at all with its ancestral progenitor system as depicted in **Figure 3** (Ashenberg & Laub, 2013; Cheng et al., 2016). In fact, researchers have shown that the artificial introduction of point mutations into TCS genes allows one to "rewire" distantly related TCSs, creating new signal transduction cascades by mimicking the evolutionary processes described above (Skerker et al., 2008).

However, as fast as bacterial evolution is, these evolutionary processes of differentiation do take time. It is therefore not surprising that TCSs which are the product of relatively recent gene duplication events may not have diverged to the point of full insulation from one another. Such systems could retain the ability to communicate with one another and may have complex signal transduction cascades involving interactions between multiple sensors or response regulator proteins. Our laboratory and others have demonstrated that in certain cases interactions between recently divergent TCSs can occur and that such interactions can be beneficial for the bacterium. In fact, there is evidence that some of these cross-regulating TCSs are important for bacterial pathogenesis, although the frequency with which such interactions occur and their overall impact on bacterial fitness is a topic of much needed additional research (Guckes et al., 2017; Kostakioti et al., 2009; Steiner et al., 2018).

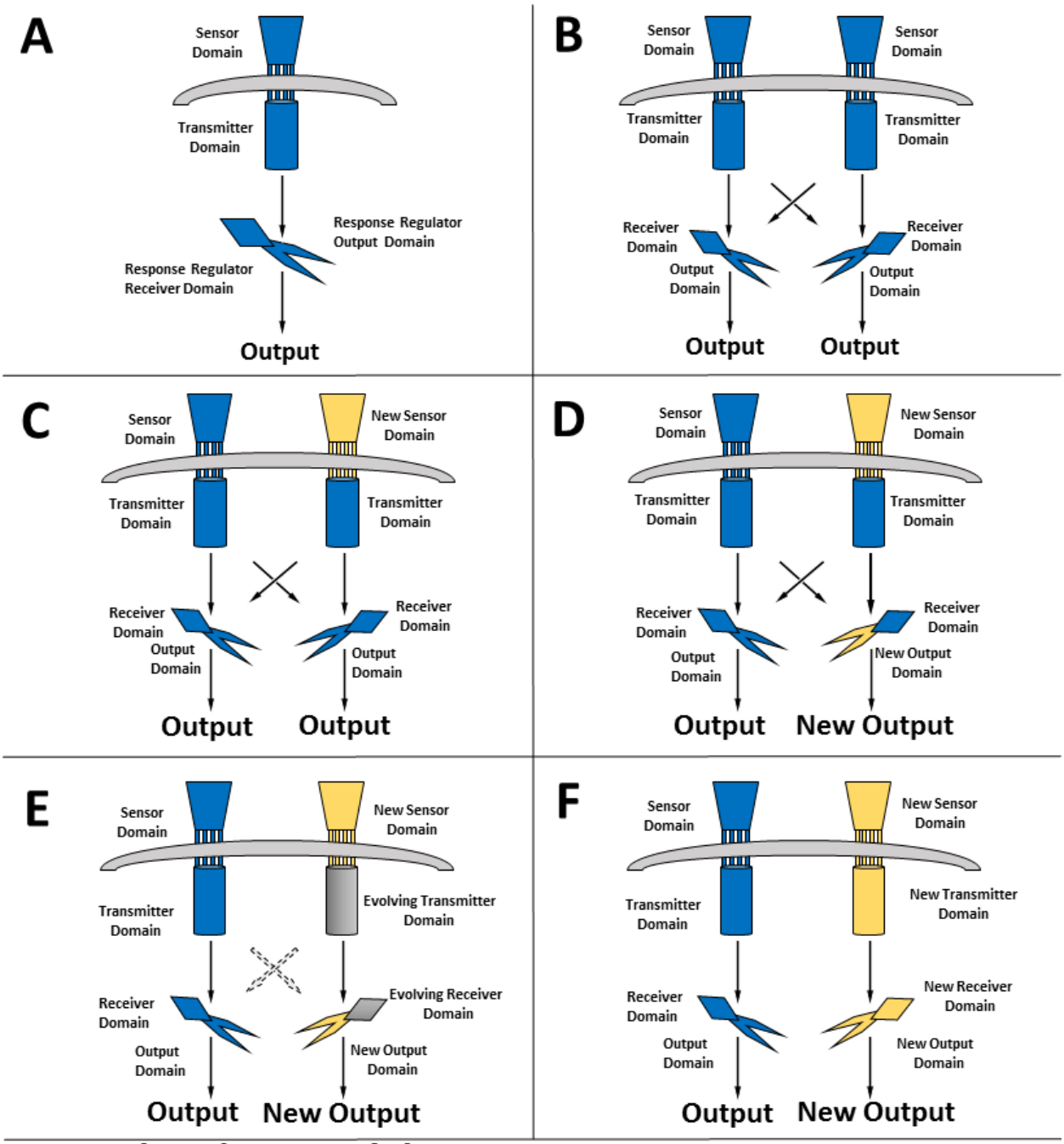


Figure 3: Evolution of TCSs via gene duplication events

A) Initial TCS with a defined input and output. **B)** Upon a gene duplication event two identical TCSs with the same input and output exist. **C)** The duplicate TCS will only be retained if it begins to rapidly differentiate itself from the progenitor system, frequently, and as depicted here, this occurs through alterations in the sensor domain. **D)** Further mutations alter the output domain of the new TCS. This system now has a newly evolved function within the bacterium but detrimental cross-talk between the two systems prevents optimal functioning and fitness. **E)** A series of compensatory mutations evolve in the catalytic domains of the sensor and regulator, cross-talk between the systems begins to occur less and less frequently. **F)** Eventually, after the evolution of alternating mutations, the new TCS and its ancestral progenitor no longer interact with one another and the systems are insulated and independent.

It is exciting to consider that some bacterial signaling cross-interactions have evolved in a way that is actually beneficial to the bacteria and that these interactions may have been retained throughout evolution. This line of thinking raises the possibility that bacterial signaling systems may be far more complex than initially believed and opens the door for future exciting research into the evolution of bacterial signaling networks that may even function as "four-component systems". Additionally, as the field develops a growing appreciation for the ability of sensor kinases to act not only as kinases but also as phosphatases (Gao & Stock, 2013; Huynh & Stewart, 2011), it becomes a very real possibility that interactions between TCSs may have evolved that allow for negative regulation across systems and/or the expansion of signal responses using different iterations of the same systems (Breland et al., 2017). Such a regulatory scheme would allow for efficient regulation of the genome and help to explain how most bacterial species harbor a comparatively small number of TCSs relative to the number of signals they must respond to (depending on species the number of TCSs in a bacterial genome can range from zero to over 200 with an "average" bacterial genome coding for ~30 TCSs (Schaller, Shiu & Armitage, 2011) .

Two-Component Systems as Potential Drug Targets

TCSs are not known to exist within metazoans and higher eukaryotes. It has been speculated by some groups that the evolutionary loss of TCSs in animals may be due to the need for longer and more stable signaling cascades which can be better provided by biochemical events such as phosphoryl group hydrolysis. In animals, signal fidelity must be maintained in the journey from the membrane to the nucleus, a task that highly labile aspartate-phosphate moieties may be poorly suited for (Capra & Laub, 2012). This theory may explain why some plants and fungi have inherited TCSs via lateral gene transfer from endosymbiotic organelles but that TCSs are, as Capra & Laub 2012 put it: "conspicuously absent" in the higher eukaryotes. Regardless, although the absence of TCSs in animals may be shrouded in evolutionary theory and speculation, the fact that

humans do not possess them makes targeting and disrupting TCSs an attractive antibacterial strategy. In recent years this topic has moved from the realm of theory and into the lab, as several groups have begun preliminary studies to assess TCSs as potential antibacterial drug targets. An example of such work is seen in the promising efforts of groups such as the Utsumi laboratory of Kindai University in Japan. The Utsumi laboratory has begun to test a variety of TCS inhibitors and has focused much of their efforts on the anti-histidine kinase compound Signermycin B. Signermycin B targets and inhibits the WalK/WalR (YycG/YycF) TCS, a TCS known to be a master regulator of cell wall metabolism and growth in certain bacteria. The Utsumi lab's work has shown Signermycin B to have noteworthy antimicrobial properties against Gram-positive bacteria such as *Streptococcus mutans*, *Staphylococcus aureus*, and *Bacillus subtilis* (Okada et al., 2010; Watanabe et al., 2012). Such studies provide strong rationale for continued research into antimicrobial compounds targeting TCSs. It is important to note here that compounds targeting TCSs do not necessarily need to be independently lethal. Given that TCSs have been implicated in antibiotic resistance mechanisms such as the production of efflux pumps or the buffering of cell walls in response to antibiotics (Gebhardt & Shuman, 2017; Kellogg et al., 2017; Sun, Deng & Yan, 2014), pharmaceuticals that disrupt these implicated TCSs could be highly valuable if used in conjunction with traditional antibiotics. A combinatorial therapy combining an antibiotic with a TCS inhibitor involved in antibiotic resistance might help to lengthen the functional lifespan of widely used antimicrobial agents. Combinatorial therapies of this sort might also be useful in reducing the required effective dose needed for some antibiotics. This is an important consideration in light of the fact that some antibiotics can cause problems such as acute kidney damage even at frequently used therapeutic doses (Bamgbola, 2016). That said, we must be careful to ensure that research into TCSs is not limited to those systems with already established links to antibiotic resistance or essential bacterial processes. By studying other systems, we not only increase our knowledge of bacterial pathogens and signaling biology, we open the door to entirely novel potential therapies. There are many TCSs that may be involved in processes such

as cell wall reinforcement, metabolism, or motility that could make for excellent combinatorial therapy targets. One can envision scenarios in-which inhibiting a TCS involved in a metabolic or cell wall process initially thought unrelated to antibiotic resistance might in fact prime a bacterium for antibiotic susceptibility. Lastly, as the field advances and generates new classes of antibiotics the potential to use TCS inhibitors alongside those new compounds should help to reduce the likelihood of rapid antibiotic resistance evolving in the bacterial population.

Given that TCSs have numerous functional domains we must consider how to best target and inhibit them pharmacologically. The conserved nature of the sensor/regulator residues and the general similarity of all TCS sensor-regulator interaction interfaces presents us with a fantastic potential target for inhibition. Because all TCSs function via a conserved mechanism of histidine to aspartate phosphotransfer, the catalytic region of TCSs provides an extremely attractive target for compound development. A compound that prevents histidine kinases and response regulator proteins from properly engaging in phosphotransfer could potentially disrupt numerous TCSs simultaneously. This is an important consideration, as the targeting of a single system in bacteria, even one important for growth or survival, can lead to the rapid acquisition of resistance in the bacterial population. By disrupting numerous systems at once a TCS inhibitor might have broad-spectrum activity and could potentially eliminate many types of bacteria with a reduced risk of drug resistance evolving. Additionally, as we come to understand the impact of the microbiome on human health it is clear that many bacteria harbored within humans are beneficial. Traditional antibiotics operate via a “sledgehammer” approach, targeting processes that are essential for all bacteria, and in the process killing both the “good” bacteria of the host microbiome as well as invading pathogens. Excitingly, pathogenic and non-pathogenic bacteria appear to have many differences in their signaling networks, raising the possibility that TCS inhibitors can be designed that disrupt pathogenic bacteria without negatively impacting the fitness of the host microbiome. Additionally, research has shown that the disruption of TCSs in pathogens does not always result in killing of bacteria, and instead can sometimes “re-wire” pathogens towards a non-pathogenic

state. TCS inhibitors show promise not only for their potential to reinvigorate old and failing antibiotics but also to open the door to the treatment strategies of the future which could potentially eliminate a pathogen threat whilst sparing the host microbiome from destruction.

The need for new antibacterial therapeutics cannot be overstated. Over the last several decades bacterial pathogens have become increasingly resistant to our antimicrobial arsenal with some pathogens acquiring resistance to numerous, or in some recent cases, all commonly used antibiotics (Meletis, 2016; Ventola, 2015; Zhi-Wen et al., 2015). Even drugs such as colistin aka polymyxin E, a powerful antibiotic which has had limited usage in humans due to harmful side effects, have not escaped the slow but steady evolution of antibiotic resistance. The bacterial gene *mcr-1,* known to generate colistin resistance in bacteria, was identified in Chinese pig farms just a few short years ago, presumably in response to colistin usage in the agricultural industry. *Mcr-1* is now being identified in patient samples not only in China but also in the USA and elsewhere (MacNair et al., 2018; Matamoros et al., 2017). Unfortunately, research into new antimicrobial strategies has not kept pace with this growing problem, and in the last few decades only a single new class of antimicrobial compounds has been discovered (Brannon & Hadjifrangiskou, 2016). This sobering reality makes it clear that we must increase research efforts aimed at identifying and creating next generation antimicrobials. As there is no way of *a priori* identifying which potential antimicrobial strategies will perform best, preliminary research into a wide-variety of plausible and feasible strategies should be encouraged, and targeting two-component systems required by pathogens for successful infection and survival represents one such approach. However, TCSs are clearly a large class of proteins and there are many different bacterial pathogens with many different TCSs. Ideally, research should be focused on TCSs that appear to be important for the pathogenesis and lifecycle of high impact pathogens.

One such pathogen of strong medical interest is uropathogenic *E. coli* (UPEC). UPEC is a wide-spread pathogen and a leading agent of bacterial infections, causing over 80% of

complicated and uncomplicated urinary tract infections (UTIs) in the USA. UTI related symptoms are estimated to cause over 10.5 million physician office visits and 2-3 million emergency department visits every year (Flores-Mireles et al., 2015). UTIs significantly impact quality of life and can occasionally be fatal if not treated appropriately; and costs associated with UTI treatments, both in direct healthcare and lost productivity, total in the billions of dollars every year. However, as troubling as these statistics are, they may not represent the biggest problems associated with UTIs. With a worldwide estimate of 150 million people being affected by UTIs every year there are tremendous amounts of antibiotics being used to treat this issue. In fact, it has been estimated by varying sources that UTIs represent either the most frequent or the second most frequent reason for antibiotic prescriptions in the USA (Flores-Mireles et al., 2015; Foxman, 2010). The substantial amounts of antibiotics used to treat UTIs are very likely a significant contributor to the accelerated frequency of multi-drug resistance in *E. coli* and other common bacteria, a link that has not escaped the notice of the World Health Organization and other international bodies (Prestinaci, Pezzotti & Pantosti, 2015). Given the importance of antibiotics in the practice of modern medicine it is important that we begin to work towards new antibacterial pharmaceuticals and coming to better understand the signaling networks of high impact pathogens such as UPEC may provide us with effective targets for those future compounds.

Signaling Systems in UPEC: Much to Be Learned

UPEC can colonize a wide variety of locations within the body and as such must possess not just the signaling systems needed to survive within the bladder, its primary site of infection, but also systems that allow it to navigate and survive the potentially lengthy journey to the bladder. UPEC can be introduced to the host via a variety of vectors, including uncommonly suspected routes such as ingestion of contaminated food, meaning that these pathogens must be able to survive the highly acidic environments of the stomach and upper digestive tract

(Nordstrom, Liu & Price, 2013; Singer, 2015). From these acidic locales UPEC can move and subsequently colonize the lower gastrointestinal tract (Guglietta, 2017; Thoma, 2017) and can eventually leave the GI tract to traverse the perineum, enter the urethra, ascend into the bladder to infect bladder urothelial cells and possibly continue upwards to the kidneys (Schwartz et al., 2011; Yamamoto et al., 1997). Once inside of these bladder urothelial cells UPEC can multiply, form intracellular bacterial communities with distinct roles and functions for different bacteria, and can elongate and escape from the hijacked epithelial cell back into the bladder lumen from which it can infect other urothelial cells ensuring a cycle of infection and evasion (Berry, Klumpp & Schaeffer, 2009; Conover et al., 2016; Hadjifrangiskou et al., 2012; Rosen et al., 2007). UPEC represents an excellent organism in which to study signaling networks in no small part due to this complicated and robust lifecycle. It is highly conceivable that targeting and disrupting the TCSs that pathogens such as UPEC need for their complicated lifestyles will reduce their fitness and help to alleviate their burden on human health.

However, although targeting and disrupting TCSs is a sound theoretical idea, many gaps in our knowledge need to be addressed before we can begin testing potential compounds against classes of TCSs. For example, fundamental knowledge elucidating *which* of the 32 TCSs in UPEC are active and critical during infection will allow us to pinpoint specific drug targets for testing. Recent work in our laboratory and in the laboratories of our collaborators has sought to assist in addressing this gap in knowledge by evaluating and characterizing the function, regulation, and importance of several TCSs in UPEC. One set of systems that I have been involved in studying are the YpdAB and BtsRS TCSs. YpdAB and BtsRS are two separate sensory systems comprised respectively of the sensor/regulator protein pairs YpdA/YpdB and BtsS/BtsR. YpdAB and BtsRS have been shown to control the downstream transcription of two genes, *yhjX* and *yjiY* respectively (**Figure 4)** (Kraxenberger et al., 2012), and both genes are predicted via *in silico* analysis to code for membrane embedded transport proteins. Work in our laboratory has demonstrated that these two downstream genetic targets are significantly upregulated in murine models of acute UTI (6hrs

post-infection) and chronic UTI (4 weeks post-infection) (Behr et al., 2017; Conover et al., 2016). Upregulation of *yhjX* and *yjiY* in murine infection models suggests that YpdAB and BtsRS are active during infection. We thus sought to define the contribution of YpdAB and BtsRS to UPEC fitness.

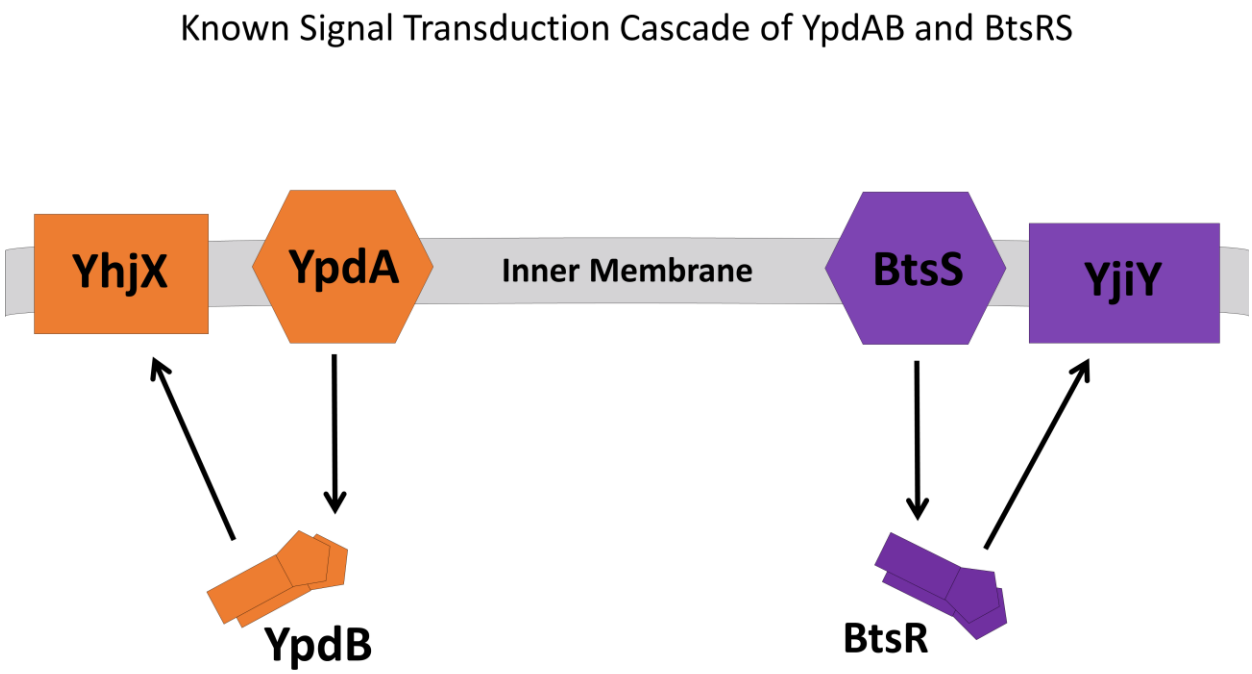


Figure 4: The YpdAB and BtsRS TCSs

Schematic depicting the YpdAB and BtsRS signal transduction cascades. The sensor proteins YpdA and BtsS interact with YpdB and BtsR respectively. The YpdAB system is the only known activator of the gene *yhjX*, which encodes for a putative membrane transport protein. The BtsRS system is the only known activator of the gene *yjiY*, which also encodes for a putative membrane transport protein. Each system is only known to have its respective gene as a downstream target.

Chapter 2

YpdAB & BtsRS Signaling

A portion of the work has been published in the journal "Journal of Membrane Biology" as Steiner et al 2018, Feb;251(1):65-74. PMID: 29374286

Receptor Theory and Microbiology: Approaching the YpdAB and BtsRS Systems

A challenging aspect of studying bacterial TCSs is that there are few systems for which a genuine ligand or activating factor has been identified. This complicates research for several reasons which are perhaps best explained by viewing TCSs through the lens of receptor theory. Receptor theory is a well-established paradigm for modeling and understanding the interactions between ligands and receptor systems. Receptor theory is frequently used by pharmacologists seeking to clarify the interactions between various ligands and a given receptor or signaling system's on/off state. Pharmacology recognizes that ligands can come in many forms such as agonists (which bind to and activate a receptor), antagonists (which bind to and do not activate a receptor), mixed agonist/antagonist compounds, and inverse agonists (which bind to a receptor and elicit the opposite response of an agonist) and receptor theory can be used to model and understand the complex interaction of these various compounds with a receptor system. Here we are interested in the basic paradigm of receptor theory: the two-state model of receptor activation. The two-state model of receptor activation views receptors as proteins with two conformation states: an inactive receptor state and an active receptor state. An agonist therefore would be a compound that forms a biochemical complex with a receptor protein and induces it to transition from its inactive state into its active state whereupon the receptor then exerts its biological function in the cell. This straightforward approach to viewing receptor systems is useful to keep in mind and leverage towards the study of TCSs.

Specifically, a receptor theory approach to studying bacterial TCSs relieves us of the burden of continually trying to fit new information into the complex framework of pathogenesis and host-pathogen interactions and lets us break apart these complex biological systems to focus on sensor histidine kinases for what they fundamentally are: transferase enzymes switched between an on and off state to catalyze a phosphotransfer event. The fact that they are embedded in the membrane and may or may not interact with one another or with other proteins in increasingly complex ways should not distract from an understanding of them and their activity through the clarifying simplicity of the two-state model. Regardless of whatever complex interactions future research into TCSs might reveal, receptor theory gives us a basic and solid framework to ground ourselves with. However, a receptor theory approach to studying TCSs also makes it clear that if we are to truly understand a given TCS, to quantitatively assess the regulation of that system and to map the interaction of sensor kinases and response regulators, we must be able to control the activation state of the sensor. Excitingly, with the YpdAB and BtsRS TCSs we have identified a few candidate ligands that appear to have activating properties and have experimentally demonstrated legitimate ligand binding with at least one of these candidates.

DRaCALA Assays Identify Pyruvate as a BtsS Ligand

Within the fields of receptor theory and pharmacology radioligand binding assays are a popular tool for assessing ligand-receptor interactions. These assays can take several forms, such as using mixtures of radiolabeled and non-radiolabeled ligands to assess the affinity and binding properties of various compounds for a receptor or to assay receptor distribution in a tissue sample (Maguire, Kuc & Davenport, 2012). However, in their most basic form radioligand assays enable researchers to test for the formation of a complex between a suspected ligand and a receptor. Incubating a radiolabeled version of a suspected ligand with cell samples or with the receptor proteins of interest should lead to the formation of a ligand-receptor complex and by then washing

away any unbound ligand a researcher can assess the degree to which the radioligand interacted with the receptor protein given the experimental conditions (Bylund & Toews, 1993). After washing away excess ligand, or purifying the ligand-bound receptors, the intensity of radioactivity present can be used as a measure of the extent to which the ligand and receptor interacted. Radioactive labeling can be doubly useful in systems such as TCSs where a known compound, in this case ATP, provides the phosphate that is used by the sensor kinase to phosphorylate and activate response regulator proteins. By providing radiolabeled ATP to these systems we can subsequently measure the levels of radioactivity in response regulator proteins as a readout of sensor-regulator interactions. This allows us to assess the activation state the studied TCS in response to a variety of conditions or ligands, as well as quantitatively assess the degree and frequency with which the sensor kinases and response regulators interact under the tested conditions. Radioligand experimental strategies clearly provide a number of tools that can be leveraged in the study of bacterial signal transduction networks.

In conjunction with our collaborators, a Differential Radial Capillary Action of Ligand Assay (DRaCALA) was used to identify pyruvate as a ligand of the BtsRS TCS (Behr et al., 2017). DRaCALA assays function by exposing proteins embedded in a nitrocellulose membrane to radiolabeled ligands. These membrane-embedded proteins can bind the radiolabeled ligand and subsequently ligands that are unbound are pulled away via radial diffusion. By using a given amount of radiolabeled ligand and then measuring the subsequent amounts “washed away” via radial diffusion vs the amounts left bound to the proteins in the membrane one can ascertain the degree to which a given ligand binds with the protein being studied. This method can be used to test a panel of ligands for their ability to interact with and bind a sensor protein. Once ligands of interest have been identified a useful method to test specificity of binding is a competition assay in which a “cold” ligand, i.e. one that is not radiolabeled, is added in increasing quantities to the reaction mixture. If the cold ligand is capable of binding to the protein then the addition of cold ligand in excess to the reaction mixture will yield a result in which the radiolabeled ligand is

unable to bind i.e. has been competed off. If done at several different concentrations prior to competing off all of the radiolabeled ligand, one can plot this data and generate useful information regarding the binding affinity of the compounds. In our testing, we identified pyruvate as the sole ligand of the BtsS sensor and in subsequent competition assays compounds such as L-Serine and Glycine were unable to compete off radiolabeled pyruvate. Related compounds such as phosphoenolpyruvate, when added at high concentration, were able to reduce pyruvate binding but notably did not induce *yjiY* expression, demonstrating that BtsS is a highly specific receptor for pyruvate. The dissociation constant for pyruvate binding with BtsS was found to be 58.6 ± 8.8 μM (Behr et al., 2017). The system name BtsRS, i.e. the sensor kinase BtsS and response regulator BtsR, was chosen to reflect this change in knowledge, representing the name Brenztraubensäure, the name pyruvic acid was given when first synthesized. In previous literature one can find this system referred to as the YehU/YehT (sensor and regulator respectively) system (Behr, Fried & Jung, 2014; Kraxenberger et al., 2012). Notably, although the downstream target of YpdAB, *yhjX*, is induced via the addition of high levels of pyruvate to media (600+ μM), as of now there is no direct data elucidating the ligand or activating stimuli of the sensor YpdA (Behr et al., 2017)

The YpdAB and BtsRS Two-Component Systems: Role and Function

The addition of pyruvate or serine to growth media has been shown to induce downstream expression of the genes *yhjX* and *yjiY* (Behr et al., 2014; Behr et al., 2017; Fried, Behr & Jung, 2013). Pyruvate and serine are both important metabolites that UPEC utilizes in a variety of ways as it progresses through the pathogenic cascade. Pyruvate is well known for its role as a precursor to the citric acid cycle whereby it can be directly converted into Acetyl-CoA to fuel aerobic metabolism. Naturally, the citric acid cycle is an important metabolic pathway for energy production in many organisms, but it is further notable in the context of UTIs, because the TCA cycle is required for *E. coli* fitness within the urinary tract. Several studies have demonstrated that

UPEC respire aerobically within the bladder (Floyd et al., 2016) and that mutants with disruptions in various steps of the TCA cycle have highly attenuated virulence (Alteri, Smith & Mobley, 2009; Hadjifrangiskou et al., 2011). Specifically, UPEC mutants lacking the gene *sdhB,* which encodes the succinate dehydrogenase iron-sulfur subunit used to convert succinate to fumarate, have a 50-fold reduction in colony forming units (CFUs) compared to Wild-Type (WT) UPEC during *in vivo* mouse models of UTI (Alteri et al., 2009) demonstrating that the TCA cycle is required for full UPEC virulence. Further research has found that disruptions to the TCA cycle can lead to several specific changes in UPEC phenotypes such as a dramatic reduction in type 1 pili expression (Hadjifrangiskou et al., 2011). This is notable because type 1 pili, a class of surface appendages expressed by Enterobacteriaceae (Waksman & Hultgren, 2009), are required for UPEC to adhere to and invade bladder epithelial cells (Mulvey et al., 1998). Thus, although UPEC is a facultative anaerobe it requires the TCA cycle in order to successfully complete its pathogenic cascade.

Serine is also a valuable metabolite that UPEC utilizes during infection (Anfora et al., 2007). Serine is scavenged by UPEC and can be shunted into several important pathways: it can be converted to pyruvate via the actions of SdaA/B (serine deaminase) or can be converted to glycine via GlyA (serine hydroxymethyltransferase) to be used in the *de novo* synthesis of purines. Previous studies in our laboratory have established a critical need for *de novo* purine synthesis during bladder infections: deletion of the *cvpA-purF* locus, which codes for the colicin V production accessory protein (cvpA) and purF (an amidophosphoribosyltransferase catalyzing the committing step to *de novo* purine synthesis), renders UPEC unable to replicate and expand within the intracellular niche of bladder epithelial cells. (Shaffer et al., 2017). Thus, without the ability to *de novo* synthesize purines, UPEC is able to invade bladder epithelial cells but is unable to successfully complete the transient intracellular expansion associated with acute infections. A TCS sensing and responding to pyruvate or serine could be playing a role in the regulation of these important processes (the interconnected nature of these metabolites in UPEC pathogenesis is detailed in **Figure 5**).

Furthermore, serine is a gluconeogenic amino acid. The TCA cycle and gluconeogenesis are intimately connected systems that are able to fuel one another in an anaplerotic fashion. In the absence of readily available carbohydrates amino acids such as serine can be directly converted into pyruvate in order to fuel metabolic processes. Interestingly, UPEC eschews typical bacterial metabolic observations and appears to preferentially utilize these amino acids even when glucose is readily available. Proteomics experiments studying the growth of bacteria in human urine have found that UPEC expresses several isoforms of di- and oligopeptide binding proteins, and these peptide binding proteins are needed for UPEC to properly colonize the bladder (Alteri & Mobley, 2015). Given the importance of proper metabolic regulation in bacterial pathogenesis it is reasonable to hypothesize that YpdAB and BtsRS, which are known to respond to pyruvate and serine and known to upregulate putative transport proteins, may be important signaling systems that help to regulate the UPEC pathogenic cascade. Additionally, as prior research had identified a potential for interconnectivity between the YpdAB and BtsRS systems in K12 *E. coli* (Behr et al., 2014; Behr et al., 2017; Fried et al., 2013), an uncommon phenomena with implications for antimicrobial drug design, we sought to investigate the function and potential interconnectivity of YpdAB and BtsRS in the context of UPEC.

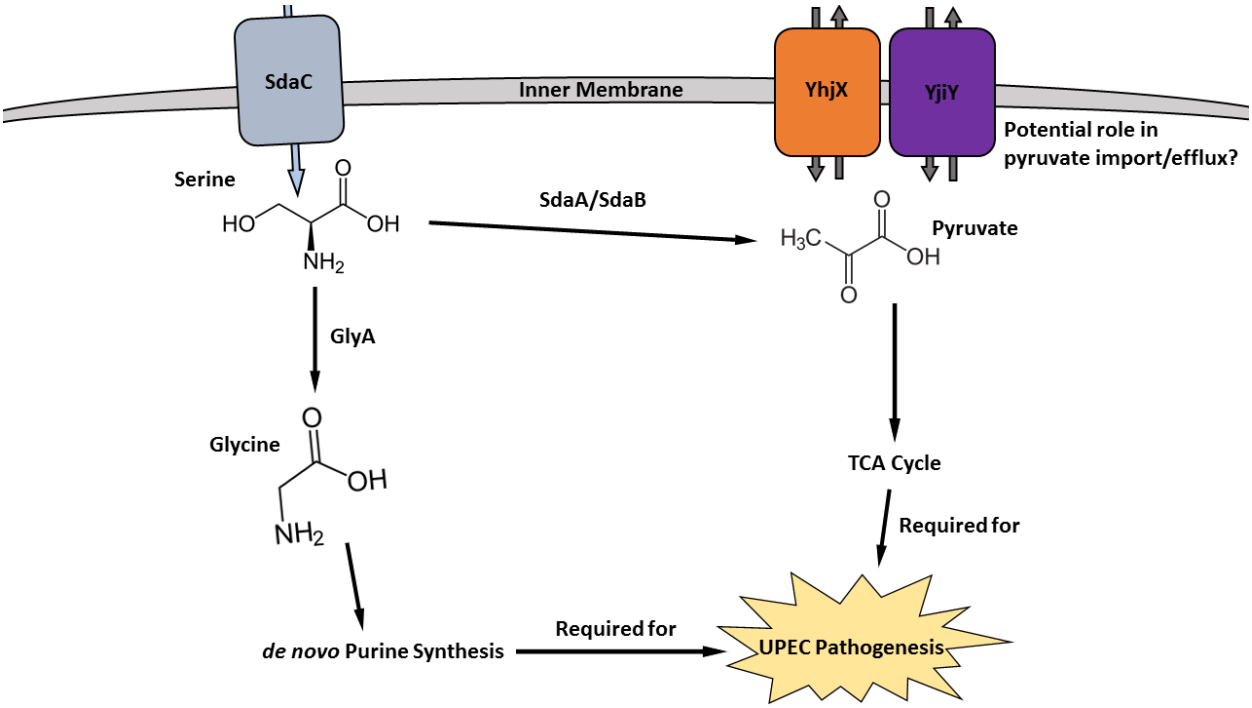


Figure 5: Role of serine and pyruvate in UPEC pathogenesis

The YpdAB and BtsRS TCSs have been shown to respond to serine and pyruvate in K12 *E. coli*. In UPEC, serine and pyruvate are linked to two processes essential for pathogenesis: *de novo* purine synthesis and the TCA cycle. UPEC can bring serine into the cytosol via the transport protein SdaC. Serine can then be converted via GlyA to glycine which can be used for *de novo* purine synthesis, a process required for UPEC to expand within host urothelial cells. Alternatively, serine can be converted to pyruvate via SdaA and SdaB. Pyruvate can be shunted into the TCA cycle which is required for UPEC to express essential adhesive pili during infection. Given that the YpdAB and BtsRS systems respond to the addition of pyruvate or serine to the media via the upregulation of the putative transport proteins YhjX and YjiY it is possible that these systems are interconnected with a larger process of metabolic regulation important in the pathogenesis of UPEC.

Non-Cognate Partner Interactions Regulate the Activity of *yhjX*

In K12 *E. coli* the downstream targets of BtsS/BtsR and YpdA/YpdB are naturally induced during *in vitro* growth in laboratory media shortly prior to stationary phase (Behr et al., 2014; Fried et al., 2013; Kraxenberger et al., 2012). To determine whether the same was true in UPEC, target gene expression was assayed using our plasmid-promoter luciferase transcriptional reporters. Promoter activity was first monitored during growth in LB under aerobic conditions at 37°C. As with K12 *E. coli*, UPEC reached maximal *yjiY-lux* and *yhjX-lux* reporter activity shortly prior to bacterial post-exponential growth phase, with expression beginning at roughly 120 minutes of growth and peaking at roughly 180 minutes (**Fig. 6a-b, filled circles**). To determine whether BtsS/BtsR and YpdA/YpdB are the sole regulators of *yjiY* and *yhjX* expression under the growth conditions tested, we constructed deletion mutants lacking both sensors (Δ*btsS*Δ*ypdA*) or lacking both response regulators (Δ*btsR*Δ*ypdB)*. In these deletion mutants, there was no downstream activity of either *yhjX* or *yjiY*, indicating that these target genes require signal transduction from one or both two-component systems (**Fig. 6a-b**, **filled squares and filled triangles**). This alteration in signaling was not attributed to changes in bacterial growth in either the double sensor kinase or double response regulator deletion mutants (**Fig. 6, black lines**).

Previous studies suggested that there is interplay between the BtsS/BtsR and YpdA/YpdB TCSs in K12 *E. coli* (Behr et al., 2014). Given the genetic diversity of the *E. coli* species, we were interested in exploring the extent to which BtsS/BtsR and YpdA/YpdB interconnectivity might exist or be altered in UPEC. In order to test the interactions between the BtsS/BtsR and YpdA/YpdB systems, we first created single gene deletion mutants lacking only one component from either system.

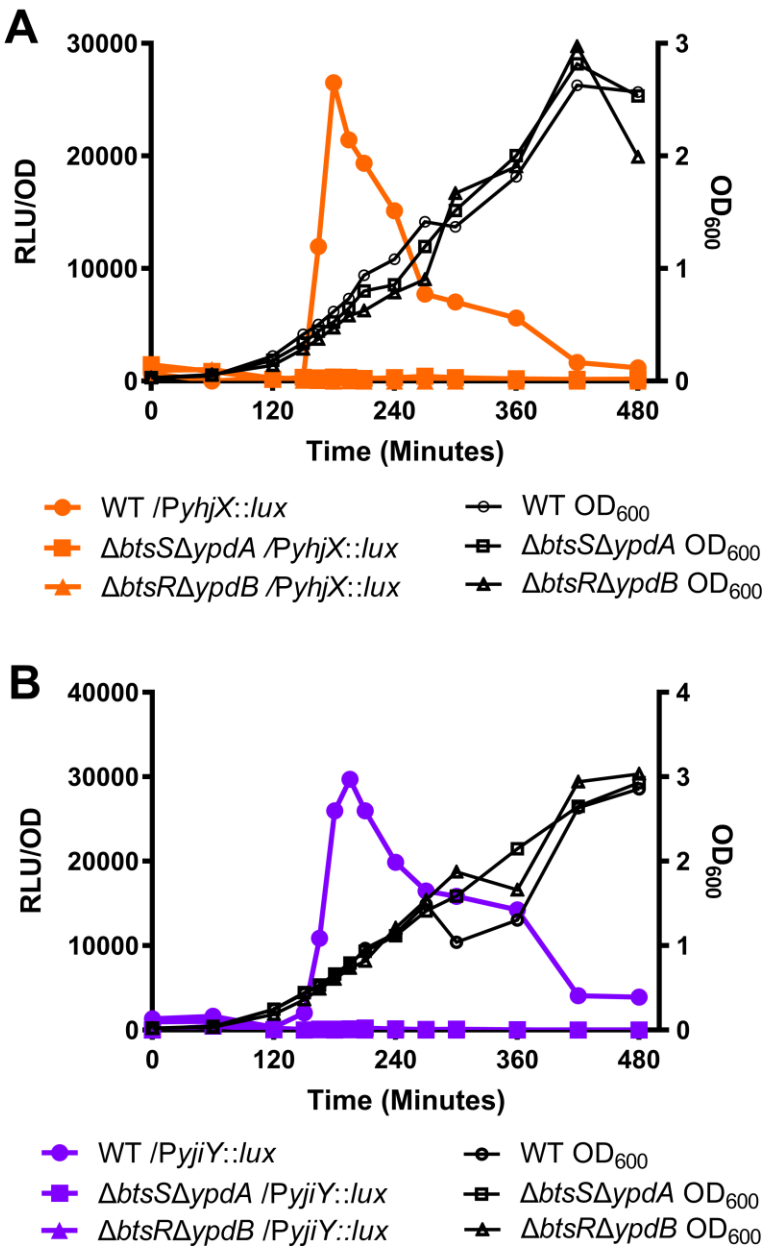


Figure 6: *yhjX* and *yjiY* activity is under the control of YpdAB and BtsRS

Measurement of optical density and *yhjX* or *yjiY* promoter activity in double histidine kinase sensor or double response regulator deletion mutants during *in vitro* growth in LB. Downstream gene activity is recorded as relative light units (RLUs). RLUs are plotted as a function of OD to normalize for variance in bacterial population. **A)** *yhjX*-lux activity and OD_{600} of deletion mutants. **B)** *yjiY*-lux activity and OD_{600} of deletion mutants. WT wild-type. Experiments were performed at least three independent times and the results of a representative experiment are shown. Published data from Steiner et al 2018.

During normal *in vitro* growth in LB, we found that deletion of either *btsS* (**Fig. 7a**) or *btsR* (**Fig. 7b**) abolished *yjiY* target gene expression, while deletion of *ypdA* or *ypdB* imparted no effect on *yjiY* expression **(Fig. 7a-b)**. These results demonstrate that in UPEC, *yjiY* is under the sole control of the BtsS/BtsR signaling system. Deletion of either *btsS* or *ypdA* sensor genes led to a substantial increase in *yhjX* promoter activity, but with distinct differences in the transcriptional surge profile (**Fig. 7c**). Although deletion of *btsS* led to dramatically increased *yhjX* driven luciferase activity, the timing of this transcriptional surge during growth was the same as in wild type (WT) UTI89 (**Fig. 7c, open circles**). Contrarily, deletion of *ypdA* abolished the transcriptional surge and produced a ubiquitous increase in *yhjX* expression (**Fig. 6c**). Testing the individual response regulator deletion mutants revealed that *yhjX* promoter activity was abolished in *ypdB* deletion mutants (**Fig. 7d**, **filled squares**), while deletion of *btsR* led to increased levels of *yhjX-lux* activity at the appropriate surge time (**Fig. 7d, open circles**). These results suggest that the BtsS/BtsR and YpdA/YpdB TCSs are both needed for proper regulation and activation of *yhjX* in UPEC.

Non-Cognate Partners BtsS and YpdB Are Sufficient to Induce *yhjX*

To investigate which components are responsible for the cross-regulation of *yhjX* expression, we constructed bacterial deletion mutants in which the genes encoding the sensor of one system (*ypdA* or *btsS*) and the response regulator of the opposing system (*ypdB* or *btsR*) were deleted, leaving only the non-cognate partners (either sensor BtsS with regulator YpdB or sensor YpdA with regulator BtsR). In the case of the BtsS/BtsR system, it appears that both the sensor BtsS and response regulator BtsR are needed for the activation of downstream gene target *yjiY*, as deletion of either of these genes ablated *yjiY* promoter activity (**Fig. 7a-7b and Fig. 8b**). These data demonstrate that BtsS/BtsR mediated regulation of *yjiY* fits the canonical model of TCS activity under the conditions tested.

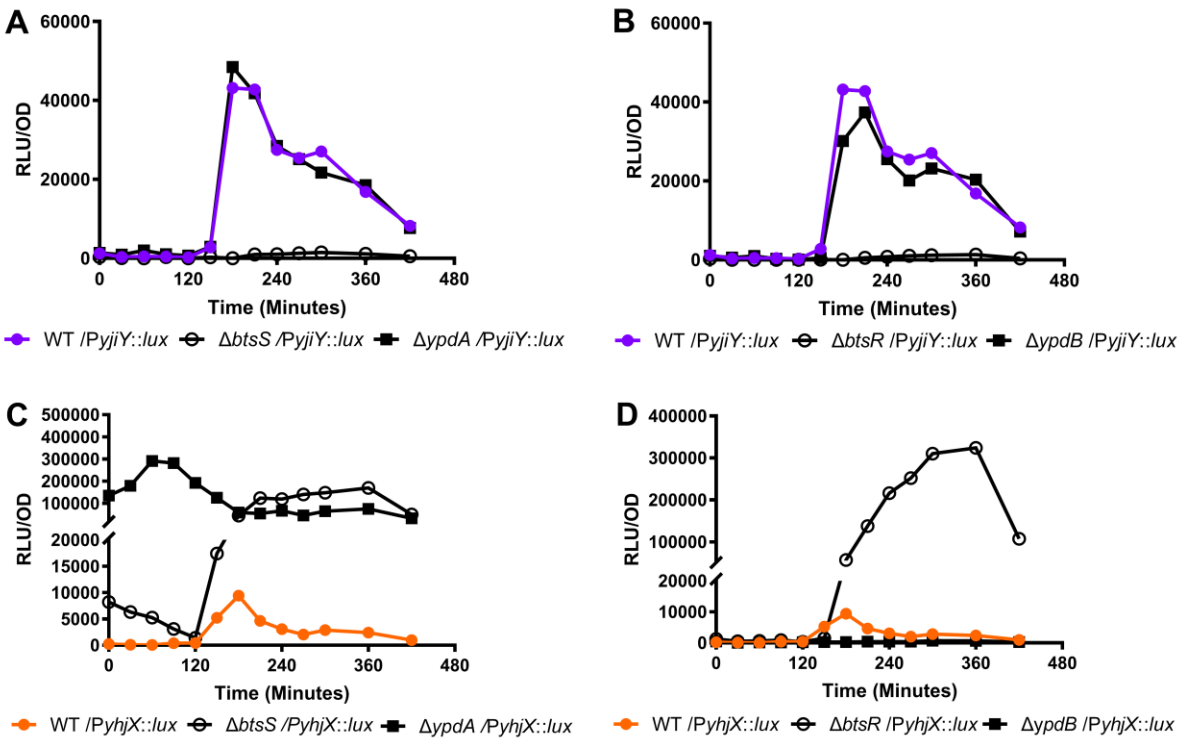


Figure 7: *yhjX* and *yjiY* activity in WT UTI89 vs isogenic deletion mutants

WT UTI89 or isogenic deletion mutants lacking components of the YpdAB or BtsRS systems were tested for *yhjX* and *yjiY* associated promoter-luciferase activity during *in vitro* growth in lysogeny broth. **A)** *yjiY*-lux activity in WT UTI89 or single histidine kinase sensor deletion mutants. **B)** *yjiY*-lux activity in WT UTI89 or single response regulator deletion mutants. **C)** *yhjX*-lux activity in WT UTI89 or single kinase sensor deletion mutants. **D)** *yhjX*-lux activity in WT UTI89 or single response regulator deletions. Experiments were performed at least three times and the results of a representative experiment are shown. Published data from Steiner et al 2018.

We next sought to determine how *yhjX* signaling might be altered in non-cognate partner mutants. We found that the Δ*btsS*Δ*ypdB* mutant had no *yhjX* activity **(Fig. 8c, black inverted triangles)**. This was in agreement with the single gene deletion experiments which suggested that *ypdB* is needed for *yhjX* activation. Contrarily, the Δ*ypdA*Δ*btsR* mutant exhibited a noticeable increase in *yhjX* activity **(Fig. 8c, half-filled hexagons)**. The Δ*ypdA*Δ*btsR* mutant fully retains the timing of the WT transcriptional surge but surpasses WT levels of luminescence. Our previous experiments had demonstrated that deletion of both sensors (Δ*btsS*Δ*ypdA*) **(Fig. 6a, filled squares)** completely ablated *yhjX* activity and that deletion of just the YpdA sensor (Δ*ypdA*) led to elevated but unregulated levels of *yhjX* activity **(Fig. 7c, filled squares)**. Taken together, these data suggest that the sensor BtsS and the response regulator YpdB interact to regulate expression of *yhjX*. The nature of this coordinated regulation might involve a number of sensor-sensor, sensor-kinase, or even system-target interactions. Thus far, our data demonstrate an interesting divergence from what has been reported in K12 *E. coli*: Notably, the single deletion analyses indicate that the expression of *yjiY* in UPEC is solely controlled by BtsRS. Furthermore, "normal" wild-type like induction and transcription of *yhjX* in UPEC requires the presence of both the BtsS sensor and the YpdB response regulator suggesting that there may be non-cognate partner interactions between these systems. This deviation between *E. coli* strains that belong to two different clades and have evolved distinct colonization strategies (Croxen & Finlay. 2010) may reflect different usage of the two TCSs to modulate bacterial homeostasis, as a function of ecological niche. It is thus possible that the "re-wiring" of signal transduction connections in UPEC may be a critical aspect of their pathogenic lifestyle. Our previous studies demonstrated that BtsS binds and responds to pyruvate (Behr et al., 2017), a metabolite that is critical for several metabolic processes important for UPEC during urinary tract infections (Shaffer et al. 2017; Floyd et al. 2015; Eberly et al. 2017; Alteri, Smith, & Mobley. 2009; Hadjifrangiskou et al. 2011). Therefore, we sought to analyze the transcriptional responses of UPEC *yjiY* and *yhjX* in the presence of pyruvate.

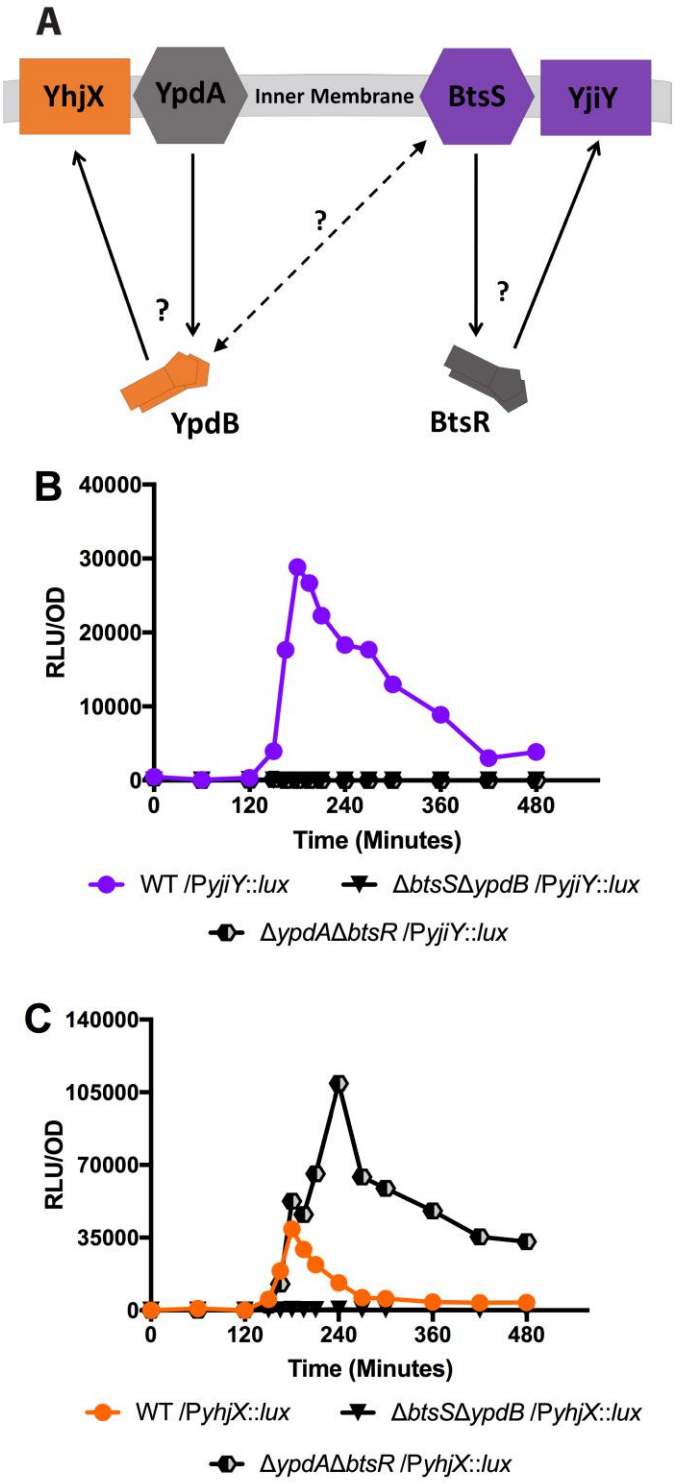


Figure 8: *yhjX* and *yjiY* luciferase activity in UTI89 mutants with no cognate histidine kinase or response regulator pairs

A) Schematic representing a Δ*ypdA*Δ*btsR* non-cognate partner mutant. Solid lines represent canonical TCS signaling pathways and dashed lines represent potential cross-interactivity. Question marks denote signaling pathway branch points of interest that were monitored for changes in downstream output between WT and the various mutants.

B) *yhjY*-lux activity in WT UTI89 and non-cognate partner mutants.

C) *yhjX*-lux in WT UTI89 and non-cognate partner mutants. Experiments were performed at least three times and the results of a representative experiment are shown.

Published data from Steiner et al 2018.

Pyruvate Signaling Through Non-Cognate Partners

Given the evidence of cross-regulation observed up to this point, we sought to determine how the addition of pyruvate would influence signaling in wild-type UPEC and in isogenic non-cognate partner deletion mutants. In these assays, the growth medium was spiked with sodium pyruvate to a final concentration of 1 mM at 120 minutes of growth, a time that coincides with the natural induction of *yhjX* and *yjiY* (**Fig. 6**). We observed in WT UPEC that pyruvate stimulation led to a considerable increase in *yhjX*-driven luciferase activity (**Fig. 9a**), while addition of pyruvate to the Δ*btsS*Δ*ypdB* mutant elicited no *yhjX* response (**Fig. 9b**). Given that BtsS is known to be a high affinity sensor for pyruvate in other *E. coli* these data suggest that in UPEC BtsS senses pyruvate, becomes activated, and induces the subsequent upregulation of *yhjX* via the action of YpdB.

Interestingly, the addition of pyruvate to the Δ*ypdA*Δ*btsR* mutant led to a sustained increase in *yhjX* activity beyond that encountered in WT strains (**Fig. 9b, half circles, Fig. 9c**). This may be explained in part by the fact that removal of YpdA (which is cognate to YpdB) and BtsR (which is cognate to BtsS) removes potential interaction partners, which can potentiate interaction between the remaining non-cognate partners. Similar observations have been made by our group in other TCS interaction studies (Guckes et al. 2013; Guckes & Breland et al. 2017). Together, our findings demonstrate the discovery of physiological interactions across the YpdA/YpdB and BtsS/BtsR two-component systems in UPEC in response to pyruvate, a known BtsS ligand, and demonstrate that all four components of the BtsS/BtsR and YpdA/YpdB systems must be present to properly regulate target gene *yhjX*.

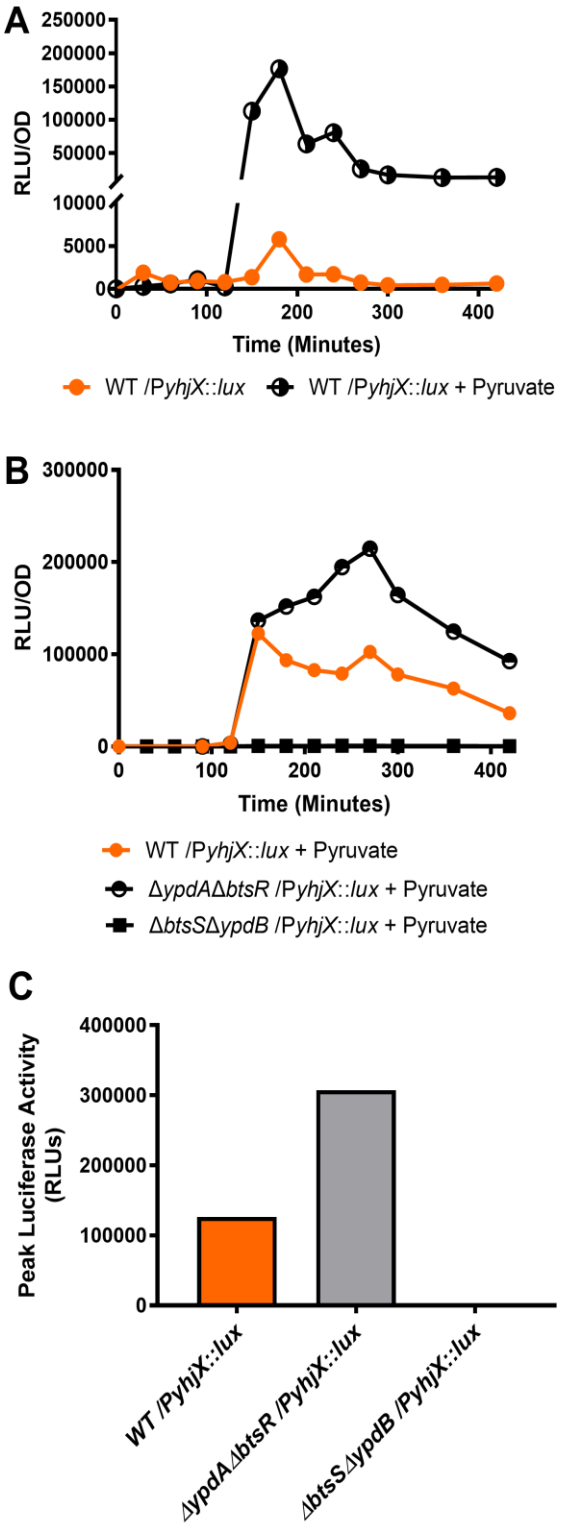


Figure 9: Pyruvate induced expression of *yhjX*-lux activity in WT and in non-cognate partner mutants.

A) Comparison of *yhjX*-lux activity in response to 1mM sodium pyruvate added at 120 min during *in vitro* growth in LB.

B) Comparison of *yhjX*-lux expression in WT UTI89 or non-cognate partner mutants with 1 mM sodium pyruvate added at 120 min.

C) Peak *yhjX*-lux RLU values observed under the same conditions as **(B)**, data from a separate representative run. All experiments were done in triplicate with representative data sets being shown.

Published data from Steiner et al 2018.

Deletion of *yhjX* and *yjiY* Deregulates Signaling

As mentioned previously pyruvate signaling plays an important role in UPEC pathogenesis. A signaling network responding to and possibly controlling the acquisition or usage of metabolites such as pyruvate and serine is therefore likely to be important during pathogenesis. In previous studies, we have reported that *yhjX* and *yjiY* are significantly upregulated in both acute and long-term murine models of UTI (Behr et al., 2017; Conover et al., 2016), suggesting that these transporters are used during infection. Computational analysis of the YhjX and YjiY proteins suggests that they are membrane-embedded transport proteins, the function of which remains uncharacterized. Deletion of either *yhjX* or *yjiY* deregulated BtsS/BtsR and YpdA/YpdB mediated induction of *yhjX* and *yjiY* (**Fig. 10**), suggesting the presence of feedback regulation by the target genes to the signaling systems. Although it was not surprising that chromosomal deletion of either downstream gene increased promoter activity of the associated gene in our promoter-luciferase fusions (**Fig. 10a**), we were excited to see that deletion of *yjiY* caused an increase in *yhjX* promoter activity in UPEC (**Fig 10b**). Indeed, when growth media was spiked with pyruvate as in previous experiments, both the *ΔyjiY* and *ΔyhjX* mutants displayed substantially greater *yhjX* promoter activity than WT under the same conditions (**Fig. 10c**), suggesting that there may be direct feedback regulation from *yjiY* to both signaling systems or perhaps that there is some level of functional redundancy between them.

None of the mutants used in this experiment displayed gross growth deficits (**Fig. 10d**) although the deletion of *yjiY* and *yhjX* was correlated with a lower final culture density. It is possible that the loss of these putative transporters causes UPEC to less efficiently utilize available resources during nutrient limited conditions. In the future, we would like to investigate whether these systems play a role in proper nutrient acquisition or usage in high-density bacterial populations such as the biofilms frequently formed by UPEC during infection.

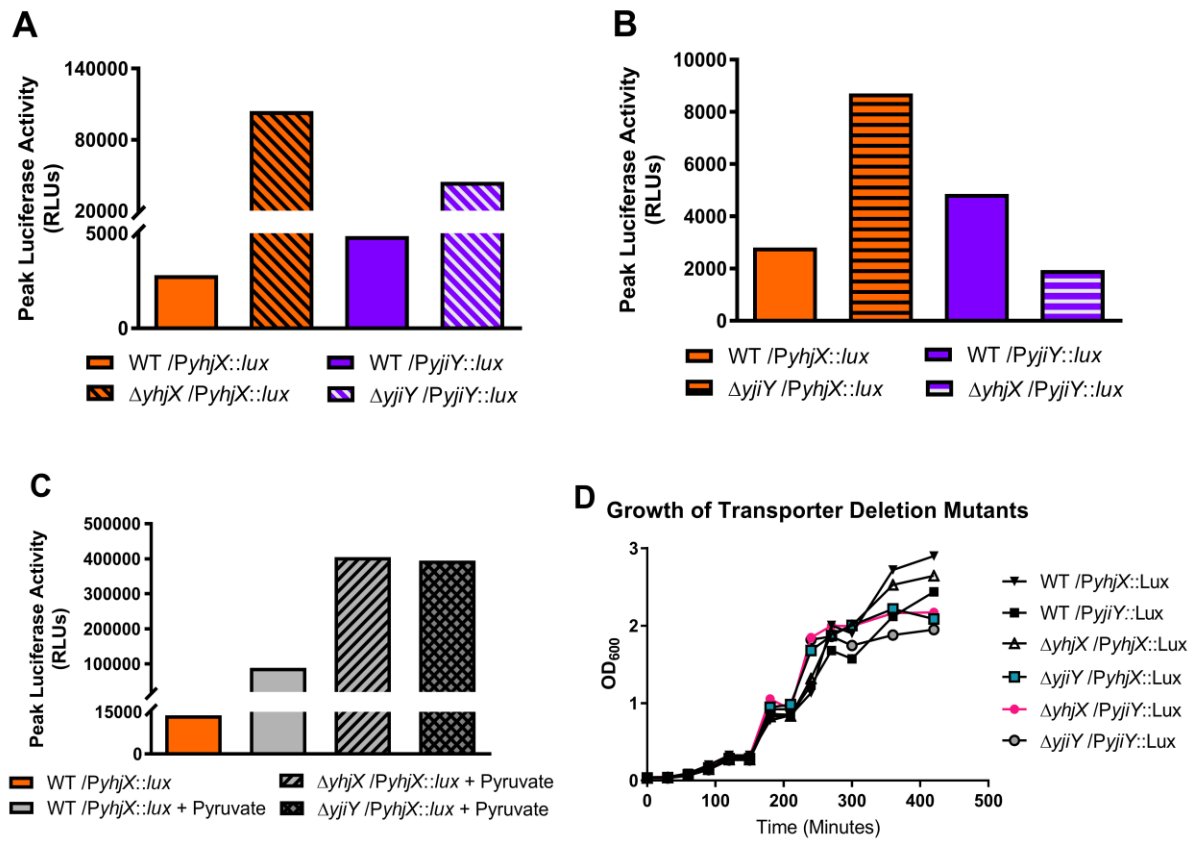


Figure 10: Analysis of *yhjX* and *yjiY* promoter activity in mutants lacking putative transporters *yhjX* or *yjiY*

A) Comparison of maximum luciferase readings for *yhjX* and *yjiY* in WT UPEC vs *yhjX* activity in Δ*yhjX* mutants and *yjiY* activity in Δ*yjiY* mutants. **B)** Comparison of maximum luciferase readings in WT UPEC vs *yhjX* activity in Δ*yjiY* mutants and *yjiY* activity in Δ*yhjX* mutants; i.e. observing whether deletion of one system's downstream target will alter promoter activity of the opposing downstream target. **C)** Analysis of maximum *yhjX*-lux activity observed in WT, Δ*yhjX*, and Δ*yjiY* strains when exposed to 1mM sodium pyruvate at 120 minutes during *in vitro* growth in LB. **D)** Measurement of optical density over time in UTI89 Δ*yhjX* or Δ*yjiY* deletion mutants during *in vitro* growth in LB. Published data from Steiner et al 2018.

The basis of the observed interconnectivity between these two systems could be due to a number of factors, and in the future we will work to understand whether the results seen here are caused by non-canonical sensor-response regulator interactions, by interactions between the response regulators themselves, or by some other means. Moving forward, we will also work to characterize and elucidate the nature of these system cross-interactions and to understand the basis of their differential activity in pathogenic and commensal *E. coli* strains. It is conceivable that uropathogens, given the limited resources typically available in the bladder environment, have evolved semi-redundant systems that work in concert to allow them to efficiently seek out and capitalize on important resources such as pyruvate. Such systems could provide us with a wealth of knowledge regarding host-pathogen interactions and pathogen competition for resources within the body as well as revealing niche specific resource bottlenecks pertinent to various types of bacteria. Proposals and experimental means to move forward with additional signal transduction studies will be detailed in Chapter 4: Future Directions.

Chapter 3

Acid Sensing in the YpdAB and BtsRS TCSs

Two-Component Systems: A Broader View of Activating Factors

In the previous chapter we explored the data supporting the assertion that the YpdAB and BtsRS systems bind and respond to pyruvate via an interconnected signal transduction cascade. However, although the downstream target of the YpdAB system, gene *yhjX*, is induced via the addition of pyruvate to media, thus far no legitimate ligand binding, either with pyruvate or another compound, has been demonstrated for the sensor YpdA. There are a number of potential explanations for this finding. It may be the case that there is a legitimate ligand for YpdA and that experiments conducted to date have not included this compound. It could be that the YpdA protein has lost its own endogenous sensitivity to stimuli and has been retained for its ability to mediate interactions with or between the response regulator YpdB and the BtsRS system, or potentially with other systems. Additionally, it could be that the YpdA protein does not bind a ligand but is responsive to other forms of stimuli, such as membrane perturbations or alterations in the proton-motive force established across bacterial membranes. The first two possibilities are very challenging to test. In the first case, if there is a ligand for YpdA that was not identified in our initial screen of likely candidates we could potentially undertake a much larger screen in the hopes of identifying a ligand. However, such a screen would be slow and expensive and there is no guarantee that the library used would actually contain a ligand or stimuli for YpdA. Regarding the second possibility, we do have data indicating that YpdA is needed for maintaining appropriate signal transduction, as deleting YpdA leads to deregulation of *yhjX* transcriptional activity. However, it would be very challenging to assert with any confidence that YpdA has no ligand or stimuli, as such an assertion is nearly impossible to validate experimentally. These experimental realities make the first two possibilities a real challenge to tackle in an affordable and reasonable time frame. The third possibility though, that YpdA does in fact respond to stimuli but that this

response is not caused via direct ligand binding but some other event, is an interesting possibility that our luciferase encoding transcriptional reporters allow us to test in a fairly high throughput and straightforward manner. When one consults the literature it becomes readily apparent that there are known examples of TCSs that function without known direct ligand binding. Some TCSs are activated by the complex interaction of numerous stimuli simultaneously as found in the CpxAR two-component system (Hunke, Keller & Müller, 2012). The CpxAR system has been implicated in multidrug resistance due to its ability to sense membrane disruptions and strengthen the bacterial cell membrane in response (Srinivasan et al., 2012). How exactly CpxAR senses these membrane perturbations is not clear, and CpxAR is not the only system with an uncertain stimulus. However, by recalling our receptor theory strategy for investigating TCSs we can envision scenarios in which alterations to the membrane, changes in proton motive force across the membrane, or pH imbalances might cause our sensor to shift from its inactive conformation to its active state. After all, sensor kinases are membrane embedded enzymes and changes to the local environment of the sensor or to the bacterial cell wall could potentially cause conformational changes within membrane embedded proteins. Indeed, the evolution of sensors embedded in the membrane that are inactive and unresponsive to exogenous ligands but undergo conformational shifts to become active in response to changing cellular conditions would be an elegant and functional strategy for dealing with the pressures and challenges of bacterial life.

A literature search for papers mentioning the *yhjX* or *yjiY* genes will turn up a published genome wide transcriptomic analysis of K12 *E. coli*. In this paper, the authors had analyzed the entirety of the K12 transcriptome in response to exposure to acid stress (Kannan et al., 2008). I was excited to see that *yhjX* and *yjiY* expression levels showed up in their transcriptomic analysis as being significantly altered in response to HCl added to growth media, with +7.4 fold and -3.2 fold differences respectively. I therefore sought to test whether *yhjX* and *yjiY* expression would be similarly altered by acid stress in UPEC.

Acid Stress Alters *yhjX* and *yjiY* Associated Luciferase Activity in UPEC

In our previous signal transduction experiments we noted that during *in vitro* growth in laboratory media there was a rapid uptick in *yhjX* and *yjiY* promoter driven luciferase activity at around 120 minutes of growth in UPEC, and a corresponding peak in activity at around 180 minutes. I wanted to test if the addition of HCl to our growth media could alter the previously observed maximal *yhjX*- and *yjiY*-lux activity we had recorded. I chose to run an *in vitro* growth experiment measuring luciferase activity as had been done in our signal transduction experiments (refer to methods for details) but spiked the growth media with HCl at 120 minutes. HCl was added to a final concentration of 15mM, chosen because this was the concentration used in the rapid acid stress transcriptomic analysis paper previously mentioned (Kannan et al., 2008). Luciferase values were compared against UPEC growing in media not spiked with HCl. The results for yhjX-lux activity in these experiments is shown in **FIGURE 11**.

These data suggest that *yhjX* can be strongly induced in UPEC via the addition of HCl to growth media. The YpdAB system is the only known regulator of the gene *yhjX* and as demonstrated in our prior experiments **(Figure 7)** alterations in the YpdAB or BtsRS systems can alter *yhjX*-lux activity substantially. It is therefore reasonable to hypothesize that the acid stress induction of *yhjX* is occurring through the control of YpdAB and is perhaps influenced by BtsRS as well. To test whether outside sensors or response regulators may be sensing acid stress and responding by the upregulation of *yhjX*, mutants were created lacking both sensor histidine kinases, Δ*ypdA*Δ*btsS*, lacking both response regulators, Δ*ypdB*Δ*btsR*, or lacking both sensors and both response regulators Δ*ypdAB*Δ*btsRS*. As can be seen in **Figure 12**, these UPEC mutants are unable to activate *yhjX*-lux activity, even when subjected to an acid challenge. This data supports the assertion that the YpdAB system is responsible for mediating *yhjX* activity in response to acid stress.

When the same acid stress experiment was performed while analyzing *yjiY* promoter driven luciferase activity we found that the addition of acid to growth media inhibited maximal *yjiY* activity in UPEC (**Figure 13)**. The BtsRS system is the only known regulator of the gene *yjiY* and as seen in our earlier signal transduction experiments deletion of either the sensor gene *btsS* or the response regulator gene *btsR* entirely ablates *yjiY* signaling, suggesting that BtsRS is necessary for *yjiY* induction **(Figure 7)**. However, given the interconnectivity between the YpdAB and BtsRS systems, I sought to determine if alterations in the YpdAB system might affect the observed *yjiY* acid stress activity. What was found is that in UPEC deletion mutants lacking the sensor gene *ypdA* this inhibition of *yjiY* activity is much weaker, with *yjiY* activity levels approaching that of WT non-acid induced UPEC (**Figure 14)**. Altogether, the data strongly suggests that the YpdAB and BtsRS systems in UPEC are sensitive to acid stress, that these systems respond to said acid stress by up- or downregulation of the genes *yhjX* and *yjiY* respectively, and that this acid stress response may involve cross-regulation between the two systems.

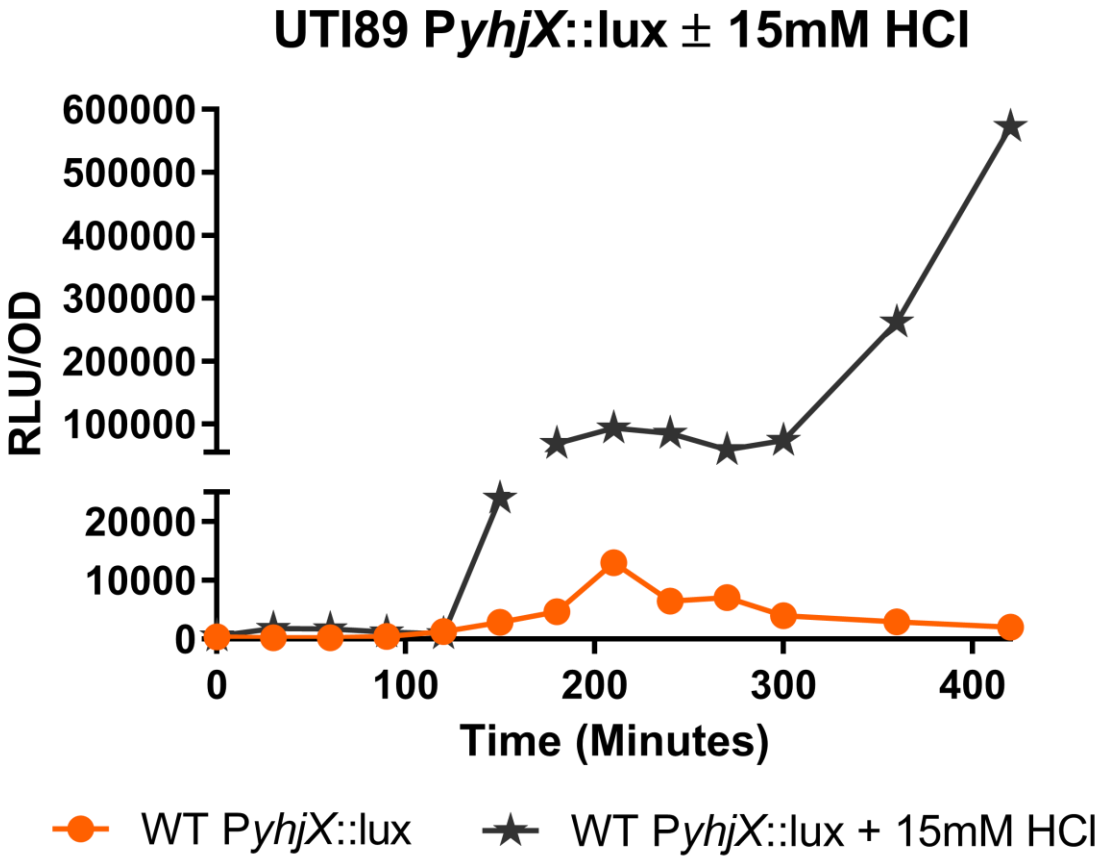


Figure 11: The addition of HCl to growth media enhances *yhjX* associated luciferase activity

When growth media is spiked with 15mM HCl at 120 minutes into *in vitro* growth there is a substantial increase in *yhjX* promoter driven luciferase activity. Experiment was performed with three technical replicates of each condition (HCl exposed and non-HCl exposed) and multiple biological replicates have been performed. Shown here is a representative experiment. Unpublished data.

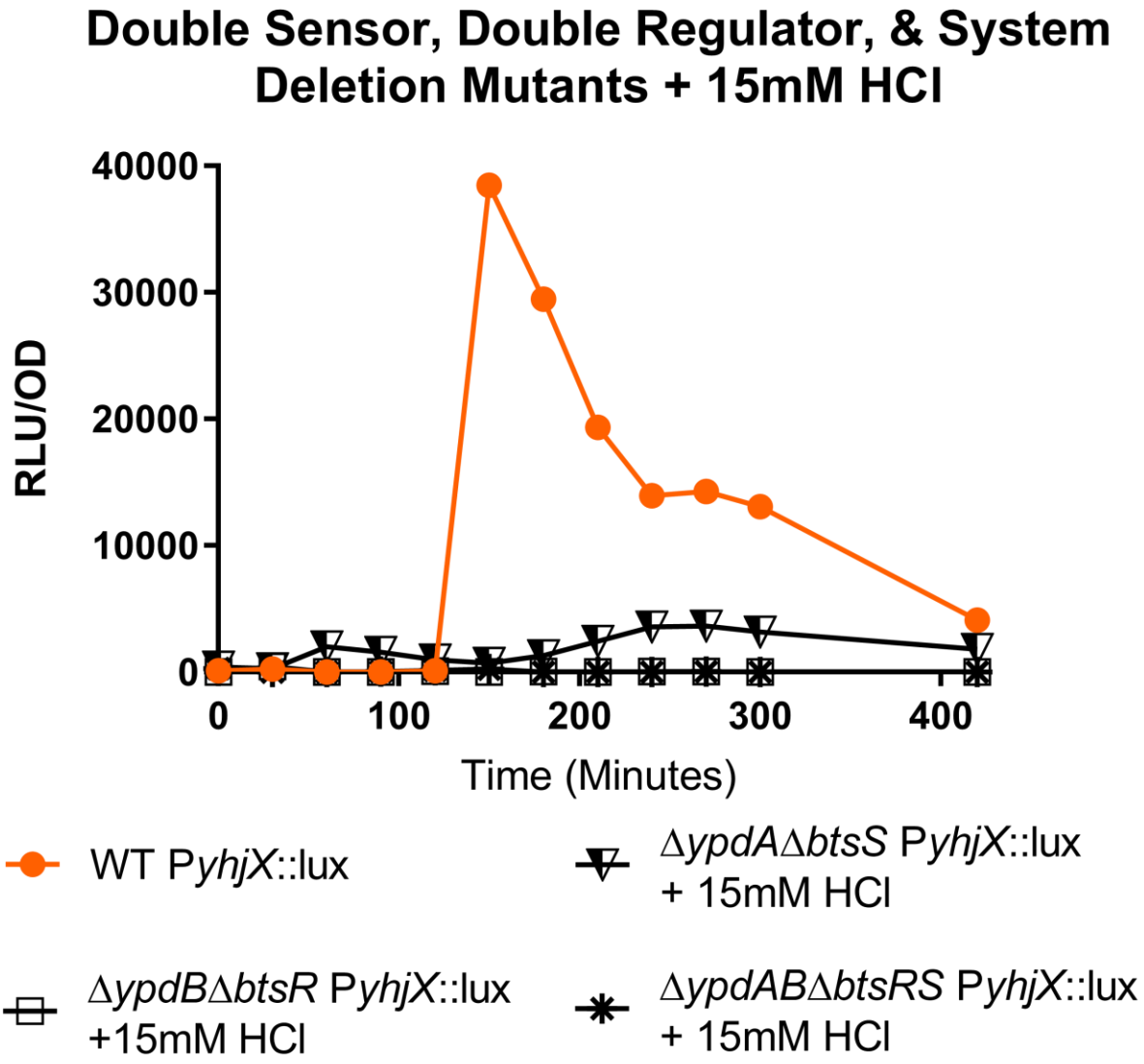


Figure 12: YpdAB and BtsRS double deletion mutants lack *yhjX* associated activity in response to HCl

UTI89 double and quadruple deletion mutants were created that lacked sensor or response regulator pairs from the YpdAB and BtsRS systems. Mutants lacking both sensor histidine kinases, Δ*ypdA*Δ*btsS*, lacking both response regulators, Δ*ypdB*Δ*btsR*, or lacking both sensors and both response regulators Δ*ypdAB*Δ*btsRS*, show little to no downstream *yhjX* promoter driven luciferase activity when growth media is spiked with 15mM HCl at 120 minutes into *in vitro* growth. Unpublished data.

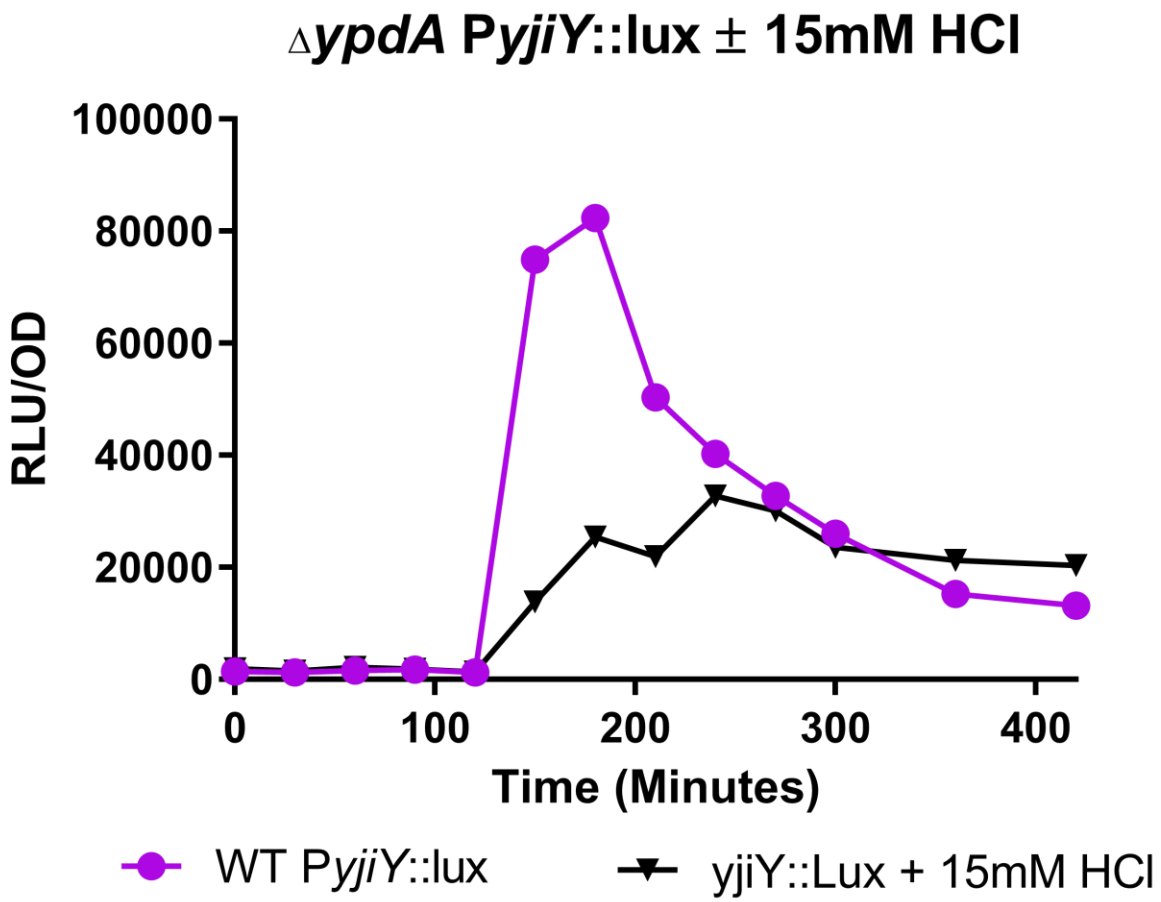


Figure 13: *yjiY* associated luciferase activity is reduced by exposure to HCl

When growth media is spiked with 15mM HCl at 120 minutes into *in vitro* growth there is a notable reduction in maximal UTI89 *yjiY* promoter driver luciferase activity. Experiment was performed with three technical replicates of each condition (HCl exposed and non-HCl exposed) and multiple biological replicates have been performed. Shown here is a representative experiment. Unpublished data.

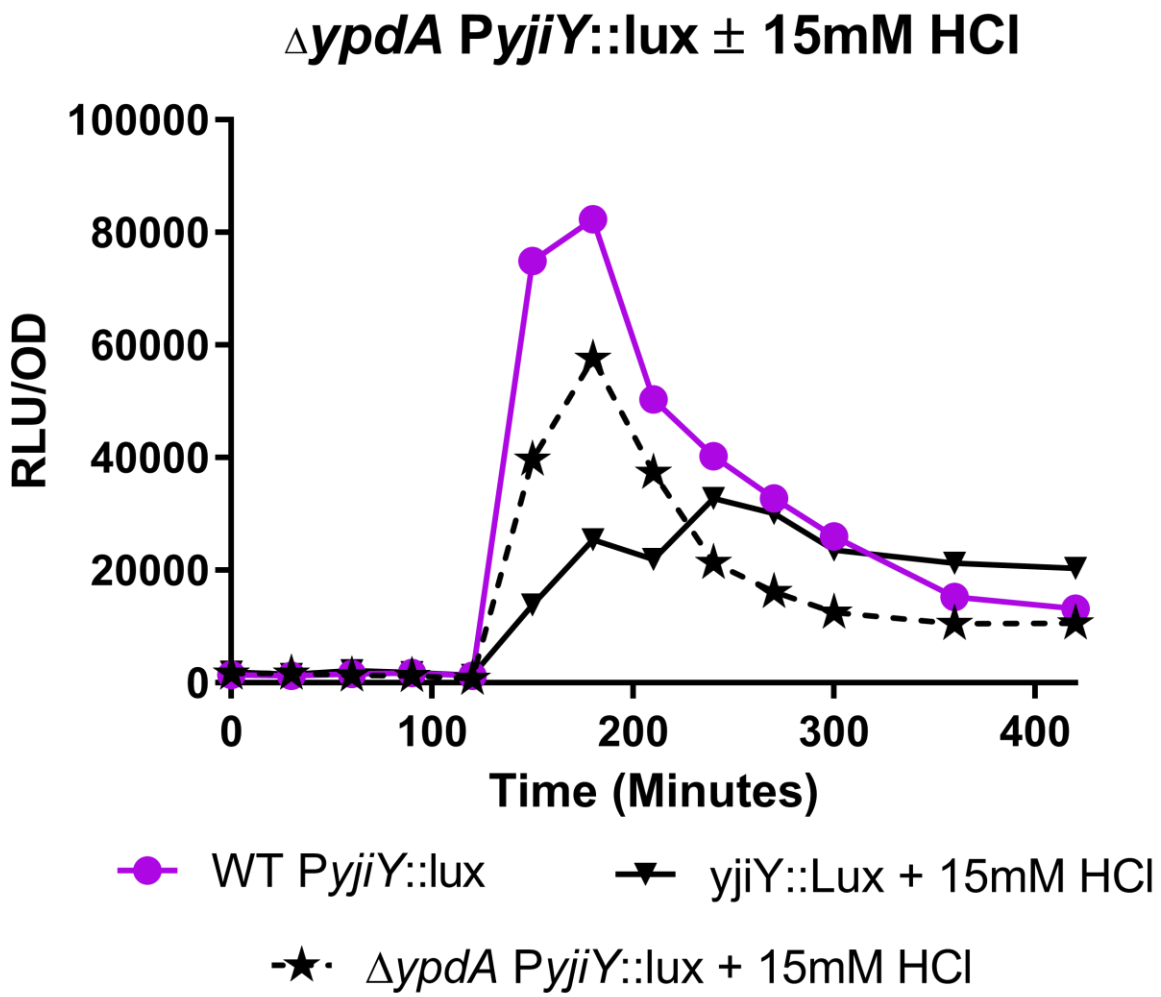


Figure 14: Deletion of *ypdA* alters *yjiY* associated luciferase activity in response to HCl

Growth media spiked at 120 minutes into *in vitro* growth lowers WT UTI89 *yjiY* associated luciferase activity. However, in UTI89 Δ*ypdA* mutants HCl repression of *yjiY* appears to be lessened, suggesting a role for the YpdA sensor in mediating the observed HCl repression of *yjiY* activity. The BtsRS system was previously the only known system known to alter *yjiY* activity, suggesting another potential cross-interaction between the YpdAB and BtsRS systems. Unpublished data.

HCl Induces *yhjX*-lux Activity in a Dose Dependent Manner

Generating a dose-response curve for a compound is a popular strategy used in the field of pharmacology to characterize the ability of a ligand or stimuli to elicit a predicted and measurable response (Tallarida & Jacob, 1979). Such a curve is generated by introducing varying concentrations of a ligand to the cells or system being studied and plotting the measurable response generated by each concentration. After finding that the addition of HCl to growth media appeared to strongly induce *yhjX* in UPEC I tested the ability of HCl to generate *yhjX*-lux activity in a dose-dependent manner. As indicated in **Figure 15** HCl, when added to a final concentration of 1mM, 2mM, 3mM, or 5mM, produces a fantastic and consistent dose response curve. This dose response appears to be saturated with the addition of 10mM HCl to the growth media. This knowledge was incorporated in future experiments where I spiked growth media to a final concentration of 10mM HCl instead of 15mM; ensuring a saturated signal response without inhibiting growth of the bacteria (refer to **Figure 16** for UPEC growth data in response to numerous acid challenges).

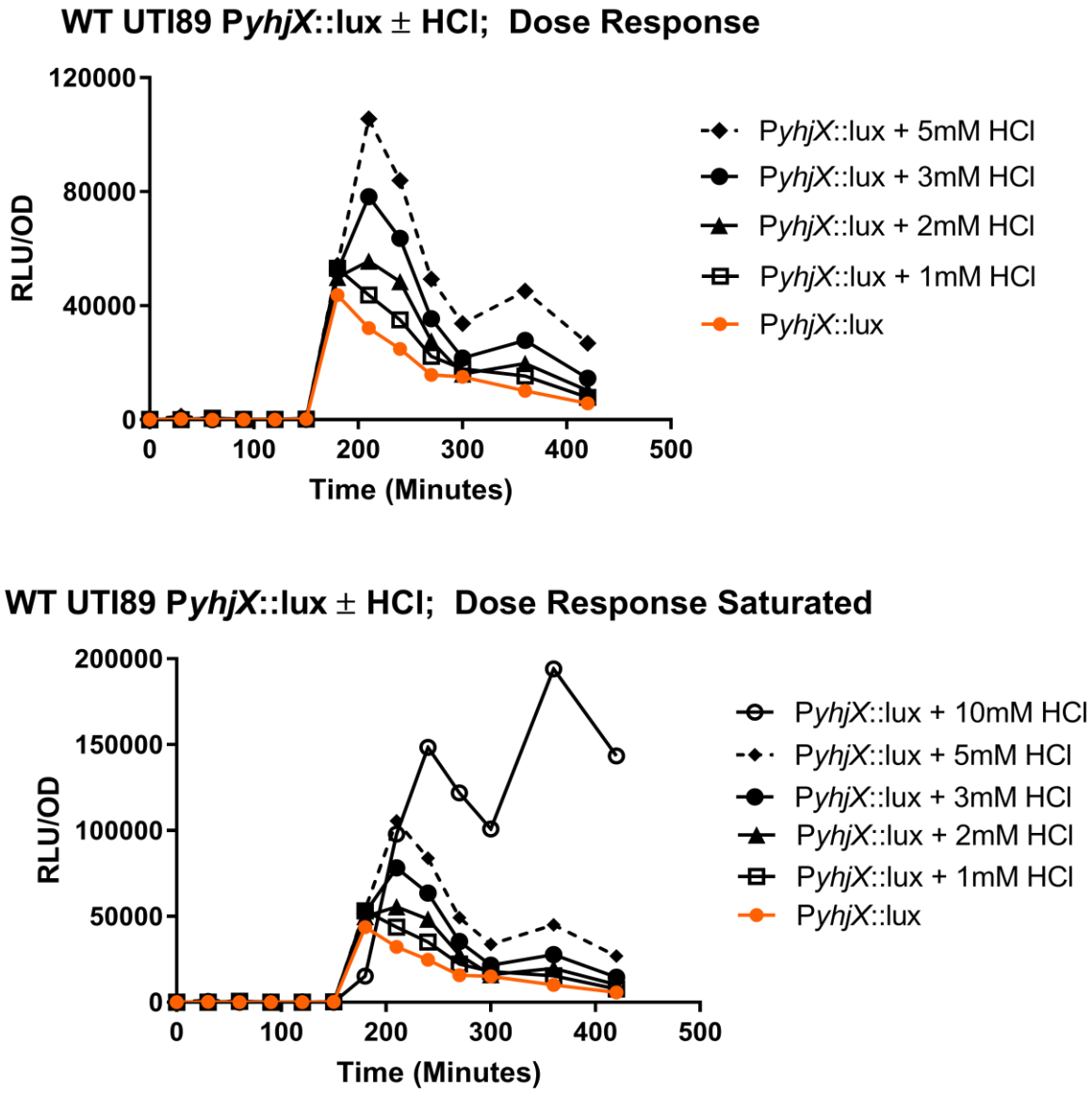


Figure 15: HCl induces *yhjX* associated luciferase activity in a dose dependent manner

HCl, when added to growth media at 120 minutes into *in vitro* growth, can induce *yhjX* promoter driven luciferase activity in a dose dependent manner in UTI89. When added to a final concentration of 1mM, 2mM, 3mM, or 5mM the WT *yhjX*-lux expression pattern is maintained but with a correspondingly higher maximal expression. When added to a final concentration of 10mM HCl saturates the *yhjX*-lux signal. Experiment was performed with three technical replicates of each condition and multiple biological replicates have been performed. Shown here is a representative experiment. Unpublished data.

Non-HCl Acids Also Induce *yhjX*-lux Activity

The data presented thus far demonstrates that the addition of HCl to growth media modulates the downstream activity of the genes *yhjX* and *yjiY*. Thus far we have termed this an "acid stress" response but there is a natural question that comes to mind when approaching this data: is the addition of HCl causing activation of downstream genes such as *yhjX* because of alterations in pH or is it because the dissociation of HCl into H+ and Cl- is creating chloride ions that can bind to or otherwise interact with YpdA? HCl was initially chosen as our experimental acid because it was the acid used in the K12 *E. coli* transcriptomic analysis paper that inspired these experiments (Kannan et al., 2008). To determine if the activity observed thus far was due to a chloride ion affect or genuine acid stress I designed an experiment to monitor *yhjX* activity in response to a battery of different acids. As the data in **Figure 16** clearly indicates, whether one adds lactic acid, acetic acid, or HCl to the growth media, *yhjX* luciferase levels are elevated far above those of UPEC growing in non-spiked growth media. It is worth noting that these various acids do appear to generate slightly different luciferase profiles and that there are even slight differences in growth patterns **(Figure 16)** depending on the acid used. It is not immediately clear why an acid such as lactic acid would generate a different growth pattern and *yhjX* signal response than acetic acid or HCl although such topics could be the focus of future experiments. The key takeaway from this experiment is that acid stress and not chloride ions appear to be the stimuli inducing *yhjX* activity.

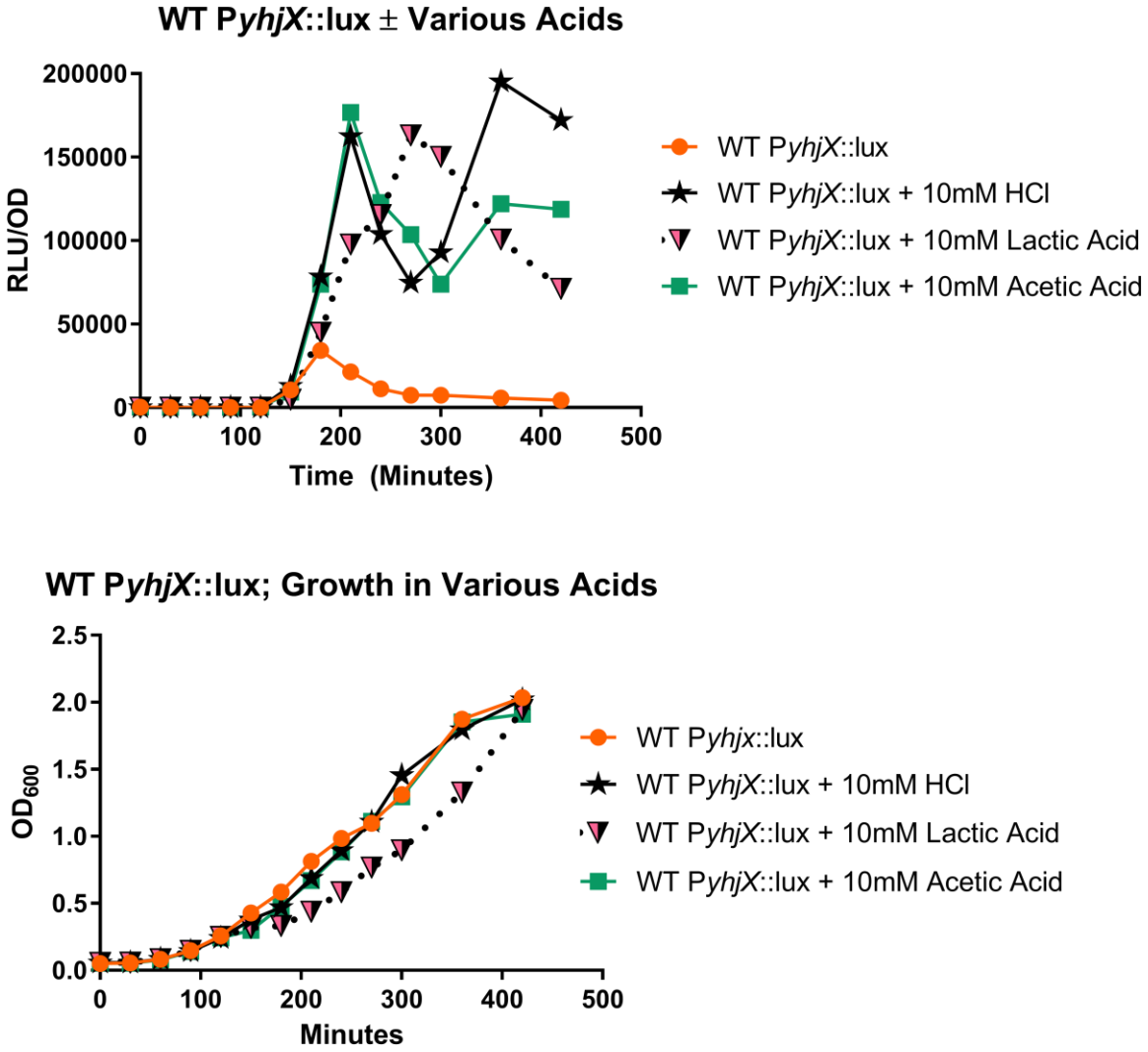


Figure 16: *yhjX* associated luciferase activity is induced by a variety of acids

In order to determine if HCl induction of *yhjX* promoter driven luciferase activity was from acid stress or a chloride ion effect UTI89 was grown *in vitro* in growth media that was subsequently spiked with either lactic acid, acetic acid, or HCl at 120 minutes into growth. All acids tested, when added to a final concentration of 10mM, were capable of inducing *yhjX*-lux activity to well above non-acid exposed levels. At the concentrations tested none of the acids prevented the bacteria from eventually growing to a max OD_{600} of ~2 and with the exception of lactic acid, where growth was slightly delayed, grew at the same rate as non-exposed UTI89. Unpublished data.

Acid Stress: Demonstrating a Stimulus Biochemically

Data suggesting that YpdAB and BtsRS sense and respond to acid stress has thus far been gathered through the use of *yhjX* and *yjiY* promoter luciferase fusion assays. However, although luciferase assays are a convenient and useful proxy for measuring downstream genetic activity they cannot demonstrate biochemically that the addition of acid to the growth media is responsible for inducing a signal transduction event in the YpdAB and BtsRS systems. While it is true that YpdAB and BtsRS are the only known systems that control *yhjX* and *yjiY*, and our data indicates that the sensors YpdA and BtsS must be present for the observed acid induction of *yhjX*, we cannot confidently state that YpdA is sensing low pH or acidic stress until we design an experiment that biochemically demonstrates that the addition of acids to growth media causes YpdA phosphorylation and activation.

In order to ascertain whether acid stress is a legitimate trigger for signal transduction in YpdAB or BtsRS we will need to utilize a radiolabeled phosphate strategy to biochemically ascertain the activation state of sensor and regulator proteins. Because ATP is the known provider of phosphate in the bacterial TCS phosphorylation activation cascade we can incubate YpdAB and BtsRS components with radiolabeled ATP, allowing us to efficiently track and measure phosphorylation levels in response to a variety of stimuli. I have begun the process of creating bacterial strains that will allow us to overproduce the various proteins of the YpdAB and BtsRS systems. By cloning each of the genes, *ypdA*, *ypdB*, *btsS*, and *btsR* into a plasmid where these genes are under the control of an inducible promoter we will be able to create membrane fractions richly populated with our sensors YpdA/BtsS and we will be able to express and purify our regulator proteins YpdB/BtsR. *In vitro* experiments with these proteins will let us test for sensor protein autophosphorylation and subsequent phosphotransfer to the response regulator proteins and allow us to ascertain whether the addition of acids to media can induce a phosphorylation cascade in YpdAB or BtsRS. At the time of this writing I have successfully cloned the constituent

genes into plasmids where they are under the control of an inducible promoter and I have electroporated these plasmids into *E. coli* strains for expression. I was not able to test their function during my time in the laboratory however and as such will address future experiments they can be used in during the future directions chapter.

Acid Stress, Pyruvate, and an Emerging Role for the YpdAB & BtsRS Systems

In the previous sections of this chapter we explored data suggesting that the YpdAB and BtsRS TCSs appear to respond to acid stress via a cohesive and interconnected signal transduction cascade. From this data two questions quickly come to mind: what role do acid resistance systems play in UPEC pathogenesis and how might the apparent acid sensing of YpdAB and BtsRS relate to pyruvate, the only established ligand of this signaling network. In the introduction we addressed some of the ways in which a bacterium may be exposed to acidic stress as it traverses the various niches of the human body. Acid stress is particularly relevant in the lifestyle of UPEC as its primary reservoir, the gut, and its site of pathogenesis and infection, the bladder, and the vaginal space which it must traverse and can colonize in women are all are low pH environments. Indeed, survival in these acidic reservoirs is very important to UPEC pathogenesis, with colonization of spaces such as the vaginal mucosa by extraintestinal pathogenic *E. coli* considered to be "a critical step toward infection" in the development of UTI's (Al-Mayahie, 2013; Obata-Yasuoka et al., 2002) [Note that in healthy women of childbearing age vaginal pH is generally ≤4.5 (Carr, Felsenstein & Friedman, 1998; Kaambo et al., 2018)] UPEC has thus evolved to thrive in acidic niches within the human body and *E. coli* on the whole have been described as "amateur acidophiles" by some researchers because the species possesses multiple well-characterized acid resistance mechanisms (Foster, 2004). Given that acidic stress destabilizes and disrupts the bacterial cell membrane, and that pH imbalances can disrupt vital intracellular processes, it is easy to understand why bacterial species might evolve multiple redundant systems for dealing

with acid stress. A bacterium, particularly one that colonizes and infects different niches with very different available nutrients and resources, benefits greatly from having multiple acid resistance systems that allow it to use a wide variety of substrates to neutralize or buffer its cellular interior and resist acid induced damage. *E. coli* possesses acid resistance systems that function on the basis of several distinct mechanisms. Some of these acid resistance mechanisms function in a roundabout way via metabolites such as glucose, which represses the formation of the cyclic AMP (cAMP) – cAMP receptor protein (CRP) complex, a regulatory complex known to repress several key acid resistance genes. *E. coli* can alleviate acid stress and directly buffer cytosolic pH by mechanisms such as coupling bacterial energy production to proton efflux or via proton dependent decarboxylation of amino acids (Foster, 2004; Kanjee & Houry, 2013).

Excitingly, a new pyruvate-mediated mechanism of acid resistance was recently discovered in *E. coli*. Specifically, this research found that glucose, whose role in acid resistance was detailed above, does not function purely via the repression of the cAMP-CRP complex (Wu et al., 2014). Rather, it is pyruvate, which we recall is a glycolysis product, that is responsible for much of the acid resistance previously ascribed to glucose. Interestingly, this pyruvate mediated acid resistance appears to function entirely independently of the repression of the cAMP-CRP complex. Pyruvate, or one of its downstream metabolites, appears to activate a small noncoding RNA, termed Spot42, and this RNA in turn activates the downstream expression of RpoS, a sigma factor known to be a central regulator of the *E. coli* stress response (Battesti, Majdalani & Gottesman, 2011). A pyruvate-mediated acid resistance system in *E. coli* is an exciting finding when considered alongside our data demonstrating that the YpdAB and BtsRS TCSs in UPEC respond to both pyruvate and acid stress. Pyruvate can be produced by bacteria from other substrates or scavenged from the environment, meaning that it is readily available in many niches, and raising the possibility that pathogens like UPEC might be able to directly modulate their intracellular pyruvate levels in order to buffer cytosolic pH and resist acid associated damage. Indeed, the aforementioned research group tested the direct relevance of intracellular pyruvate

levels on acid resistance by shutting down pyruvate-acetate efflux in an *E. coli* strain. The researchers observed that the resulting intracellular accumulation of pyruvate directly enhanced acid resistance in the tested *E. coli* (Kannan et al., 2008). Interestingly, the downstream gene targets of YpdAB and BtsRS, *yhjX* and *yjiY*, have no known function but *in silico* analyses suggest that they are membrane embedded transport proteins. It is very exciting to consider the possibility that YpdAB and BtsRS may be the mechanism, or perhaps are one of several mechanisms, by which intracellular pyruvate levels in *E. coli* are controlled to mediate acid stress.

Chapter 4

Future Directions & Discussion

YpdAB and BtsRS: Some Answers, Many Questions

The data presented throughout this thesis has hopefully provided an interesting and insightful examination of the interconnected YpdAB and BtsRS TCSs along with their potential role in UPEC pathogenesis. However, as is oftentimes the case in science an experiment or set of experiments frequently produces more questions than answers. Throughout the course of studying the YpdAB and BtsRS TCSs our laboratory and our collaborators have uncovered many further questions regarding the regulation, signal transduction, and ultimate function/purpose of YpdAB and BtsRS in UPEC. With so many potential research directions perhaps the easiest way to examine where our knowledge of YpdAB and BtsRS is lacking is by assessing the experiments that were planned but that I was unable to finish during my time in the Hadjifrangiskou laboratory.

Future Experiments: Radiolabeled Phosphotransfer

The first set of experiments that I believe should be worked towards, and that I think are truly necessary to continue moving forward in our study of YpdAB and BtsRS, are the radiolabeled phosphotransfer experiments discussed in chapter three. As mentioned there, all of the evidence provided in both my publication (Steiner et al 2018) and within this thesis for interconnectivity and cross-regulation between YpdAB and BtsRS is ultimately indirect. My interconnectivity data was generated by using genetic mutants lacking components of the YpdAB and/or BtsRS systems followed by the measurement of luciferase expressed under the control of luciferase promoter fusions for *yhjX* and *yjiY*, the only known downstream targets of YpdAB and BtsRS. These data demonstrate time and time again that deletions in one of our systems can cause obvious and

substantial alterations in downstream promoter activity associated with and under the control of the opposing system. However, although our data strongly suggests that there is cross-regulation between the YpdAB and BtsRS signal transduction cascades we do not have direct biochemical evidence that components between the systems can engage in phosphotransfer or otherwise interact with one another. Likewise, although we have shown that the addition of acids to growth media during *in vitro* growth alters *yhjX* and *yjiY* associated promoter activity, and we have demonstrated experimentally that deletion of YpdAB and BtsRS genes can ablate or attenuate acid induced alterations in *yhjX* and *yjiY* activity, we have not yet directly demonstrated that acid stress leads to phosphorylation/dephosphorylation of either of the sensors or alters phosphotransfer to either response regulator.

Using radiolabeled ATP to observe autophosphorylation of a histidine kinase sensor and subsequent phosphotransfer to a response regulator protein is a well-established strategy for demonstrating legitimate signal transduction in the TCS field. Our laboratory has used radiolabeled ATP strategies to great effect in the past, with these types of experiments underpinning much of our knowledge of the interconnectivity between the QseBC and PmrAB TCSs. I have created plasmid constructs that harbor *ypdA*, *ypdB*, *btsS*, and *btsR* under a pBad inducible promoter. In order to continue moving our research forward these constructs can be used to express and then purify the various proteins of the YpdAB and BtsRS systems for use in radiolabeled phosphotransfer experiments.

The pBad promoter is induced via the addition of arabinose to growth media (Guzman et al., 1995), and the optimal concentration of arabinose for each gene will need to be empirically identified. Too much arabinose may lead to overproduction of a protein, which can negatively impact cellular health and growth, and too little arabinose can lead to inadequate expression and too little protein for radiolabeled phosphotransfer experiments. Overexpression of histidine kinase sensors in particular can oftentimes be stressful for bacteria because histidine kinases must

be inserted into the bacterial membrane, causing cellular membrane stress, and because TCSs function best when the sensor and response regulator maintain a regular stoichiometric ratio. Overexpression of response regulators is not usually as damaging to bacterial fitness *in vitro* because response regulators are typically inactive and are located within the cytosol rather than needing to be inserted in the membrane. That said, the pBad promoter is a fairly tight regulator of its downstream genes and with the right growth conditions, and concentration of arabinose, successful expression should be very feasible. Purification of the proteins should, in theory at least, be fairly straightforward. The plasmids I have cloned the YpdAB and BtsRS genes into will affix a C-Terminal polyhistidine tag (6x His) on the proteins when they are made. These polyhistidine tags should allow the proteins to be purified by the use of commercially available affinity chromatography columns. As with the expression of the proteins these steps will likely require experimentation and troubleshooting on the part of the researcher undertaking them but the payoff will be to move our understanding of these systems forward considerably.

Once the proteins have been expressed and purified there are many experiments that can be done. The following are several ideas that I would recommend pursuing:

1) Establish baseline levels of autophosphorylation for the YpdA and BtsS sensors in the absence of stimuli; how active are these sensors *in vitro* while in growth media
2) Establish baseline levels of phosphotransfer to YpdB and BtsR from either sensor in the absence of stimuli; how active are these response regulator proteins *in vitro* while in growth media
3) Quantify the extent to which pyruvate, acid stress, or other stimuli can induce autophosphorylation of either YpdA or BtsS *in vitro*
4) Determine whether the addition of stimuli alters the “normal” signaling cascades established in part 2, i.e. does the addition of a stimulus alter the frequency with which either sensor interacts with either response regulator

5) Incubate phosphorylated response regulator proteins with either sensor in the absence of exogenous ATP to determine whether the sensors display regulatory phosphatase activity; i.e. do the sensors possess the ability to inactivate either response regulator
6) Use racemic mixtures of the YpdA/BtsS sensors and YpdB/BtsR response regulators to determine whether the protein species, when in the presence of one another, display activity different than the baselines previously established and the stimuli responses previously observed; i.e. do these proteins work together to regulate the signaling cascade

These proposed experiments should provide plenty of data for the generation of new questions and should help to inform future hypotheses of the nature and regulation of the YpdAB and BtsRS systems.

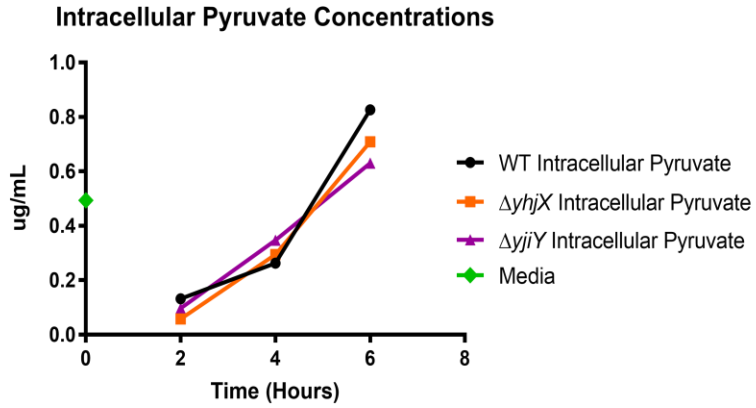
Future Experiments: Acid as a Stimulus for the YpdAB & BtsRS Systems

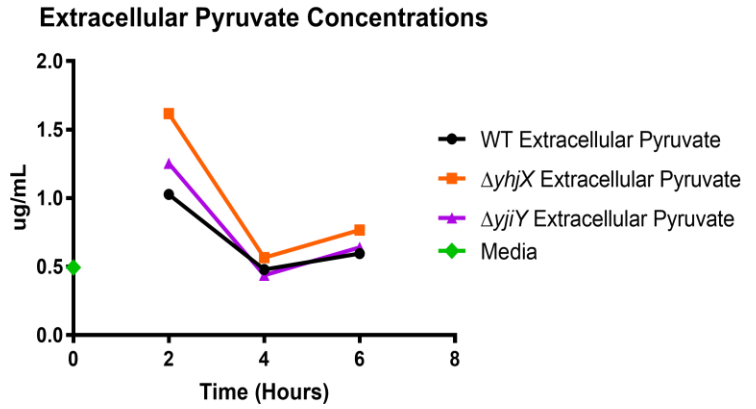
Exploring the role of these systems in acid sensing and acid resistance would be the next logical direction to take this work. There are several key experiments that could be done to survey the potential role of these systems in the UPEC acid response. Firstly, given the putative transport role for the YhjX and YjiY proteins it makes sense to assess whether these are indeed transporting molecules in/out of UPEC and whether these substrates are involved in intracellular pH homeostasis. This line of experimentation seems very promising because when we reflect back on the data presented in Chapter 3 we see that the two systems respond divergently to the addition of acid to the growth media. When acids are added to media during *in vitro* growth we see a substantial increase in *yhjX* associated luciferase activity. However, the addition of acid to media during growth led to a reduction in *yjiY* associated luciferase activity. It is possible that the expression of these two genes are inversely regulated because they function in tandem to control the bi-directional flow of metabolites into/out of the cell. Given the recent studies discussed in Chapter 3 whereby pyruvate was identified as the critical element mediating one *E. coli* acid resistance system, and our data clearly demonstrating that both pyruvate and acid stress alter

yhjX and *yjiY* signaling, it is possible that YhjX and YjiY are involved in importing or exporting pyruvate or other related metabolites into the cell in order to combat a drop in intracellular pH. One can envision a potential hypothetical in which BtsS binds pyruvate and activates its downstream gene *yjiY* under normal cellular conditions, perhaps even mediating efflux of pyruvate when it is abundant and not needed, but that when YpdA becomes active under conditions of high acid stress the signal transduction cascade is rewired to instead favor the import of this metabolite by upregulating *yhjX* and in doing so controlling intracellular pH through the aforementioned acid resistance mechanism.

Conclusively identifying the substrates shuttled by these putative transporters will be difficult, however, analyzing macro level intracellular vs extracellular concentrations of potential substrates is less difficult. Deletion mutants lacking *yhjX* or *yjiY* have already been created, and we are in the process of creating a double *yhjX* & *yjiY* deletion mutant, making it feasible to grow these mutants *in vitro* and to then assess intracellular and extracellular quantities of pyruvate, serine, or other substrates of interests via mass spectrometry, ELISA, or some other technique. Indeed, I have already performed two test runs wherein WT, Δ*yhjX*, and Δ*yjiY* UTI89 were grown in laboratory media and samples of the intracellular vs extracellular milieu were gathered at various time points. The results of these experiments are shown in **Figure 17**. These experiments were largely proof of principle, testing the feasibility of our methods for intracellular extraction (as detailed in the methods section), establishing a baseline level of pyruvate in our media, and ensuring that our samples were useable for mass spectrometry. The results are promising, with the Δ*yhjX* and Δ*yjiY* mutants appearing to have a somewhat different pyruvate acquisition phenotype. Future laboratory members should hopefully be able to push this leg of the project forward without substantial alterations in the methods we have developed and hopefully future work will be able to test mutants such as the Δ*yhjX*Δ*yjiY* double mutant I was unable to successfully create.

The Δ*yhjX*, Δ*yjiY*, and double Δ*yhjX*Δ*yjiY* mutants should also be useful for a number of experiments outside of substrate movement assays. With these deletion mutants we can rapidly move forward on experiments designed to test the importance of these genes in the UPEC acid response. Particularly, given the notable increase in *yhjX* associated luciferase activity when acid is added to growth media it would be very interesting to see what happens to intracellular pH levels, and to the ability of UPEC to grow in acidic conditions, with *yhjX* deleted. Likewise, it would be smart to include system deletions such as Δ*ypdA*, which has been shown to massively increase *yhjX* associated luciferase activity, and quadruple Δ*ypdA*Δ*ypdB* Δ*btsS*Δ*btsR* mutants, which completely ablate *yhjX* and *yjiY* activity during *in vitro* growth. These experiments would allow us to ascertain the potential effects of systemic deregulation or shutdown on UPEC acid resistance. An example experiment might be to challenge UPEC growing *in vitro* with the addition of acids to determine whether the various UTI89 mutants detailed above have a weakened ability to grow in conditions of acid stress. This would be done best by adding acid to the growth media during the exponential growth phase of the bacteria, a rationale that becomes clear when one considers *when* the various acid resistance systems of *E. coli* are utilized. Reflecting back on the pyruvate acid resistance mechanism we have discussed we recall that this system was initially thought to be glucose mediated. This is important, as glucose mediated acid resistance is the only acid resistance mechanism that is known to be active during exponential growth in *E. coli* and in general *E. coli* are considered to be able to withstand strong acid challenges of ~pH 2.5 or lower only when in stationary phase (Castanie-Cornet et al., 1999).





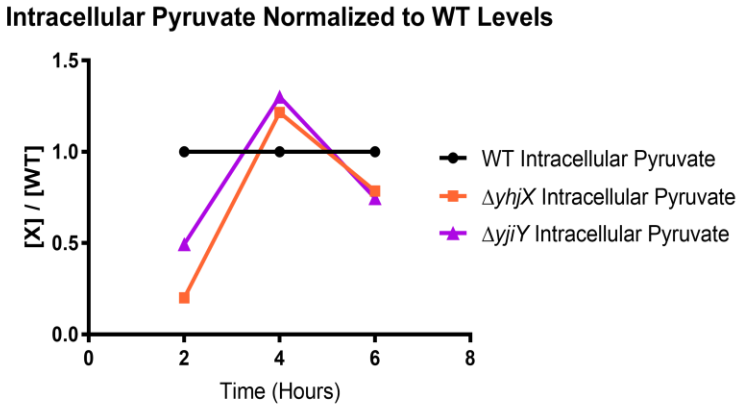


Figure 17: *yhjX* and *yjiY* deletion mutants display slightly altered pyruvate acquisition patterns.

WT UTI89, Δ*yhjX* UTI89, and Δ*yjiY* UTI89 were grown in lysogeny broth *in vitro*. Samples were gathered at the indicated time points and intracellular and extracellular pyruvate was quantified via single ion monitoring mass spectrometry.

Pyruvate levels in the media were initially recorded at 0.5µg/mL, as indicated. Both WT UTI89 and the mutants tested appear to excrete high levels of pyruvate into the media during the first 2 hours of growth. This is followed by an intake of pyruvate that between hours 4 and 6 causes intracellular pyruvate levels to reach concentrations higher than the starting media conditions.

Interestingly, when intracellular pyruvate levels in the mutants are compared to WT we see that there is a distinct biphasic acquisition of pyruvate in WT that is linearized in the Δ*yhjX* and Δ*yjiY* mutants. Normalizing intracellular pyruvate levels to WT levels highlights this difference in pyruvate acquisition. This data suggests that the YhjX and YjiY proteins may be involved in acquiring or balancing intracellular pyruvate levels throughout growth. The two proteins may function together, explaining why the loss of either appears to alter the pyruvate uptake pattern observed in WT UTI89. I was unsuccessful in creating a Δ*yhjX*Δ*yjiY* mutant but such a mutant may provide valuable insight.

Acid resistance systems requiring the decarboxylation or use of vital amino acids would be detrimental to fitness during times of rapid bacterial growth. However, freely available excess quantities of glucose can represses the formation of the cyclic AMP (cAMP)-cAMP receptor protein complex, contributing to acid resistance, and we also know now that much of this glucose mediated acid resistance is also occurring via pyruvate (either as a glycolytic product or independently scavenged). With this glucose and pyruvate mediated acid resistance *E. coli* is able to continue growing through moderate acid stress even when other acid resistance systems are shut down. This means that by exposing UPEC to acid stress during exponential growth we should be minimizing the influence of other acid resistance mechanisms and thus any differences in growth or survival in our mutants should be primarily attributable to the impact of the YpdAB and BtsRS systems on pyruvate associated acid stress resistance.

Experiments of this type have the added benefit of being fairly easy to perform. Plating samples of bacteria for CFUs is an accurate and rapid way to determine bacterial cell populations. Samples can simply be taken at a variety of time points before and after the addition of acids to determine what impact low pH has on the ability of various mutant populations to survive acid stress. For example, a gradient of acid concentrations could be applied to the various YpdAB and BtsRS mutants and it would be straightforward to quantify the bacterial population before and after moderate to severe acid stress. With our plate reader and 96 well plate assays we have another tool for easily measuring bacteria growth. Another experiment that could help to ascertain if YhjX and YjiY are involved in intracellular pH management would be to analyze growth rates and final CFUs of UPEC growing in media that has been acidified from the start. WT bacteria, although they may have their growth slowed or perhaps reach lower final culture densities compared to their growth in non-acidic media, should be able to hit log phase growth and rely on their glucose and pyruvate mediated acid resistance systems to keep the population expanding. However, if YhjX and YjiY are playing a role in mediating this process we would anticipate seeing a marked reduction in growth rates and perhaps in final culture density for our

mutant strains. These experimental setups and others could be used to probe and examine the functioning of the YpdAB and BtsRS systems as they relate to UPEC acid stress response.

Acid Sensitivity: Intracellular pH and UPEC Fitness

The casual relationship linking acidified growth media to bacterial cell death is presumably an overly acidic intracellular pH. If the putative transporters YhjX and YjiY are indeed functioning to balance intracellular concentrations of pyruvate or related metabolites, and if this function is related to buffering the intracellular pH, we would anticipate that our mutants lacking *yhjX* and *yjiY* should display a lower intracellular pH in response to acid stress than the WT bacteria. There are a number of ways in which one could conceivably test this functional relationship. If I were designing the experiments I would try to utilize one of the numerous commercially available intracellular small molecule or nanoparticle based fluorescent probes that can be taken up by cells and used to detect shifts in pH via emission spectra changes. There are many such probes and it may take some experimenting and troubleshooting to determine which ones will work best in UPEC. However, once the appropriate probes have been identified they carry the tremendous advantage of allowing for high throughput and easily interpretable results. Our laboratory already utilizes an in-house Molecular Devices plate reader for high throughput and high quality analysis of luciferase luminescence as well as ELISA assays. These devices are easy to configure and would allow us to design straightforward experiments wherein a 96 well plate containing our various bacterial strains of interest can all be incubated with the fluorescent pH probes, all exposed to the same levels of acid stress, and then intracellular pH can be easily ascertained by analyzing the emission spectra recorded by the plate reader. In the last several years these intracellular probes have become a popular and reliable way of measuring intracellular pH in eukaryotic fields and I believe they would be effective in a microbiology context as well.

Indeed, some researchers have begun to use them in precisely this context although their popularity remains lower than that found in other fields.

It is worth touching upon the role of acid stress *in vivo* once again. We recall that UPEC can colonize a variety of niches in the body, such as the GI tract, where it may be exposed to considerable acidic stress. However, the niche most relevant to uropathogens is, unsurprisingly, the bladder. An interesting epidemiological fact pertaining to UTIs is that diabetics suffer from UTIs at a rate approximately double that of the general population. At first blush one might ascribe this to glycosuria in diabetic patients, but we must recall that UPEC preferentially utilizes amino acids for fuel even in the presence of glucose. Indeed, this preference makes sense evolutionarily when one considers that in healthy humans urine typically does not contain abundant glucose. What other factors then might explain the ability of uropathogens like UPEC to thrive in diabetic patients? As it turns out, diabetic urine is frequently more acidic on average than non-diabetic urine and diabetic urine contains higher concentrations of many compounds and metabolites; very notably serine is one such metabolite (Sasaki, Sato & Maruhama, 1988). Thus, UPEC has evolved the ability to thrive in the bladder niche despite the acidic stress and relative lack of nutrients therein and is particularly well suited for survival in the even more acidic diabetic bladder niche where nutrients such as serine and other branched chain amino acids are abundant. Interestingly, when we reflect on the interconnected nature of these metabolites (refer to **Figure 5**) we are reminded that serine can be converted to pyruvate via the action of a serine deaminase reaction. If we consider this reaction in biochemical terms we are turning the serine into a pyruvate and a byproduct: ammonia, a potent base. Is it possible that UPEC scavenges serine from the bladder environment, converts it to pyruvate and ammonia, and in doing so fuels both the TCA cycle needed for pathogenesis and buffers its intracellular pH against acid stress? Such a system would be an extremely elegant mechanism for survival and growth within the bladder and I would like to see future experiments aimed at characterizing the role, if any, for ammonia in UPEC acid resistance. Monitoring intracellular pH in mutants that lack serine

deaminase genes *sdaA/sdaB*, that lack *yhjX* and *yjiY* (which may be involved in shuttling pyruvate out of the cell after the deaminase reaction occurs), or that have other related genes knocked out could elucidate a new potential mechanism of acid resistance. Clearly, although UPEC is not a "professional" acidophile, it has evolved very robust and efficient systems that allow it to survive in a wide variety of inhospitable conditions. It is possible that YpdAB and BtsRS, along with their genetic targets *yhjX* and *yjiY*, play an important role in mediating these important cellular processes.

YpdAB & BtsRS: Role in Genetic Regulation of Acid Resistance

Lastly, the final topic that I would attempt to rapidly pursue is that of genetic regulation and understanding how the YpdAB and BtsRS systems fit into the broader picture of acid stress regulation in *E. coli*. The immediate question that comes to mind is what the functions of the putative accessory proteins YpdC and YehS are (recall that the BtsR/BtsS proteins were previously annotated as YehU/YehT). YpdC and YehS represent two proteins of unknown function whose genes appear to be located within the operons of YpdAB and BtsRS respectively. What these proteins do is unknown, but we have created genetic deletion mutants lacking *ypdC* and *yehU* and I have conducted preliminary experiments to see if these mutants have altered signal transduction activity. The data is inconclusive however and more experimentation will need to be done before anything can be confidently stated about these proteins. Interestingly, in previous studies where these proteins have been analyzed it has been noted that overexpression of *yehS* leads to upregulation of the gene *iraP,* which functions to prevent proteolysis of RpoS, the master regulator of acid stress in *E. coli*. What other possible relationships exist between the YpdABC and BtsRS/YehS systems and the broader *E. coli* stress response? Previous studies have indicated that the cAMP Receptor Protein (CRP) (whose role in acid stress we outlined in chapter 3) is involved in regulating *yjiY* in K12 *E. coli* (Kraxenberger et al., 2012). Specifically, these studies identified *yjiY* expression as being dependent on cAMP-CRP, but it is worth noting that these

studies were performed in laboratory adapted non-pathogenic strains of *E. coli*, and work in our laboratory is finding that UPEC does not follow this same pattern of regulation. UPEC appears to eschew much "common knowledge" of bacterial metabolic preference and there may be substantial differences that have evolved between pathogenic and non-pathogenic strains of *E. coli*. Future work on these systems should help to clarify the extent to which such regulatory differences exist. Clearly, there are many directions one could go in to begin studying the regulation of the YpdAB and BtsRS systems. I would propose two sets of experiments: a series of protein-DNA binding experiments and then a series of experiments to test the relationship between the YpdABC and BtsRS/YehS systems and the RpoS regulon.

The first set of experiments I would attempt would be a series of chromatin immunoprecipitation sequencing assays (ChIP-seq) to identify the genomic binding sites of the YpdB, BtsR, YpdC, and YehS proteins (the two response regulators and two accessory proteins respectively). Now, there are several ways in which one can ascertain DNA-protein relationships both *in vivo* and *in vitro* and sometimes alternative techniques can be more efficient and useful than ChIP-seq, such as when the DNA/promoter regions of interest are already known. For example, if I wanted to determine if YehS or YpdC could interact with the RpoS regulon it may be more efficient and to the point to perform an Electromobility Shift Assay (EMSA) wherein purified proteins are exposed to radiolabeled PCR generated DNA fragments of interest. Running an EMSA makes it easy to identify DNA that has not bound any proteins vs DNA that has been bound based on whether or not there is a "shift" in the gel. In fact, I would recommend performing said experiments for RpoS associated regions of interest because they provide a direct and easily interpretable set of protein-DNA association data. However, I would recommend the ChIP-seq experiments in addition to the more focused EMSA experiments because they may identify entirely unexpected and unpredicted functions for our response regulators and our accessory proteins. These ChIP-seq experiments have the ability to potentially demonstrate a relationship between our system proteins and known acid resistance genes as well as a potential to

demonstrate relationships with our proteins and genes of unknown function; which can then be further investigated generating additional questions and knowledge of interrelated acid resistance systems in UPEC.

Along with all of this I would also be interested in exploring the regulation, activation, and signal transduction of the YpdAB and BtsRS systems in a physiological medium/model more applicable to human health than simply growing in LB media. We previously published data demonstrating that *yhjX* and *yjiY* are upregulated in murine models of UTI (Behr et al., 2017). However, I have performed experiments wherein *yhjX* and *yjiY* signal transduction is monitored whilst bacteria grow in human urine as opposed to LB. In some cases, such as *yjiY* promoter driven luciferase activity, the data is inconclusive and further work will need to be done. However, in other cases there appears to be substantial attenuation in the activation of these downstream gene targets, as seen when comparing *yhjX*-lux values between bacteria growing in LB with those growing in urine (**Figure 18**). If *yhjX* is known to be upregulated in murine models of UTI but does not appear to be active when UPEC is growing *in vitro* in human urine, where within the bladder niche, and when during pathogenesis, is the significant upregulation of *yhjX* occurring? Would the observed attenuation of *yhjX*-lux activity be the same if the bacteria had been grown in diabetic urine samples instead of healthy urine samples? This data raises many questions that could drive many interesting future experiments.

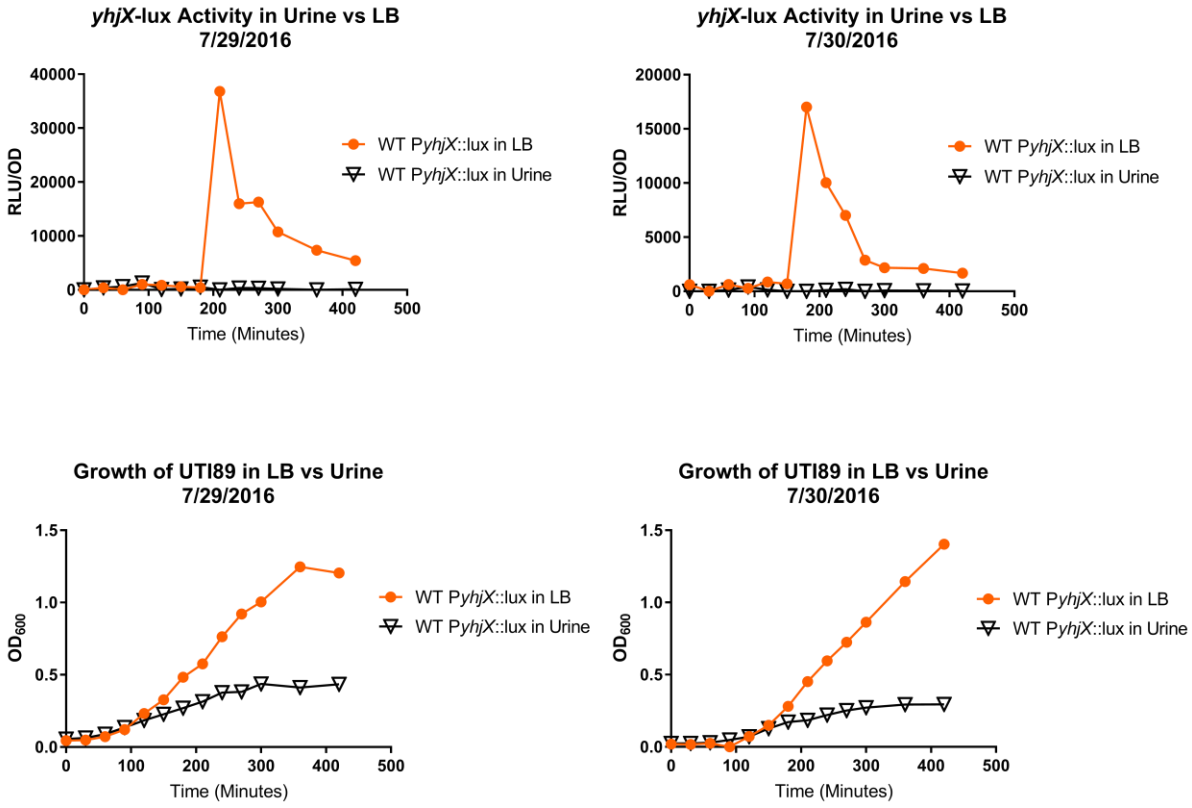


Figure 18: *in vitro yhjX*-lux activity in LB vs Urine

Measurement of *yhjX* promoter activity in WT UTI89 during *in vitro* growth in LB or urine. Downstream gene activity is recorded as relative light units (RLUs). RLUs are plotted as a function of OD to normalize for variance in bacterial population. Two independent experiments are shown. Cultures were grown overnight, three technical replicates per experiment were seeded to an $OD_{600}$of 0.03 into 96 well plates, and then OD_{600} and RLUs were measured at the indicated time points as the bacteria grew while shaking at 37c. As can be seen, *yhjX*-lux activity is substantially lower in WT UTI89 growing in urine. UTI89 growing in urine does have slower growth that reaches a lower final optical density but the difference in growth rates does not appear to sufficiently explain the difference in expression levels, particularly given that *yhjX*-lux values here are plotted as a function of optical density. Unpublished data.

Materials and Methods

Bacterial Strains and Constructs

The parent strain used in all analyses is cystitis isolate UTI89 (Mulvey, Schilling & Hultgren, 2001). Isogenic deletion strains lacking *ypdA* (UTI89_C2712), *ypdB* (UTI89_C2713), *btsR* (UTI89_C2397), *btsS* (UTI89_C2398), *yhjX* (UTI89_C4087), *yjiY* (UTI89_C5057) and permutations thereof were created using the λ-Red recombinase method of Murphy & Campellone (Murphy & Campellone, 2003). The primer sets used to make the constructs are listed in Table 1. Promoter-luciferase transcriptional reporters were created previously (Fried et al., 2013; Kraxenberger et al., 2012). Luciferase constructs were introduced into the different UTI89 strain backgrounds via electroporation. "Hyper-competent" cells were prepared for electroporation, as described in (Hadjifrangiskou et al., 2012).

Growth Conditions

Bacterial cultures were inoculated from freezer stocks and grown shaking overnight at 37°C in LB (Thermo Fisher LB Miller). Strains carrying the luciferase reporter or empty luciferase plasmid controls were grown in media containing 50 □g/mL gentamycin. The same growth conditions were used for all the reporter assay experiments. For experiments in which pyruvate was added as the stimulus, a stock concentration of 50 mM pyruvate was used and added to media as indicated below to a final concentration of 1 mM.

Transcriptional Reporter Assays

Transcriptional reporter assays were conducted in a manner similar to previous *in vivo* expression studies of the YpdA/YpdB and BtsS/BtsR TCSs (Behr et al., 2014; Fried et al., 2013). In detail, overnight cultures were normalized to a starting OD_{600} of 0.03 and were seeded into sterile polystyrene 96 well black plates with clear flat bottoms. Each well was loaded with 180 μL

of sample and a minimum of three technical replicates was conducted per strain per condition. Likewise, three wells were loaded with 180 µL of blank LB media as a background control of errant luciferase reading. Once all strains were seeded into the plate, initial readings of OD_{600} and luciferase activity were recorded and the plate was incubated at 37°C shaking. Luminescence levels are reported as relative light units (RLU). Readings were taken in a Molecular Devices SpectraMax i3 plate reader at 30-minute time intervals, over a period of 5 hours, followed by two additional readings, at 6 h and 7 h respectively. For experiments in which pyruvate was added as a stimulus, pyruvate was introduced directly following the 120-minute time point reading at a final concentration of 1 mM. Luciferase and growth density readings were exported and organized in Microsoft Excel, RLU/OD values were calculated, and then transferred to Graphpad Prism 7 for graphing and analysis. For assays involving the use of acid or pyruvate 0.5M stocks were created prior to beginning experiments. Experimental setup is the same as a standard transcriptional reporter assay

Intracellular vs Extracellular Pyruvate Quantitation

Bacteria were grown overnight and seeded 1:200 into 25mL of growth media. 250mL glass flasks were used to ensure plentiful gas exchange during growth. At time points of interest 1mL of the bacterial milieu was removed and placed into an Eppendorf tube. Samples were spun down for 10 minutes at 13,000xg and the supernatant, representing the extracellular portion of the milieu, was transferred to a new tube to be flash frozen on dry ice and stored at -80c until analysis. Chilled 50% methanol was added to the pellets and vortexed for 15 minutes to rupture the bacterial cell membranes. These samples were then centrifuged for 10 minutes at 13,000xg to spin down insoluble materials and the supernatant, representing soluble cytosolic factors, was transferred to a new tube to be flash frozen on dry ice and stored at -80c until analysis. Concurrently to these two processes a sample of the growth media milieu is to be taken and serially diluted for CFU plating. This allows one to normalize intracellular and extracellular

values obtained to the amount of bacteria in a given sample. Samples were then taken to the Vanderbilt Mass Spectrometry Core where LC/MS was used to quantify intracellular and extracellular pyruvate concentrations in comparison to a reference pyruvate standard.

Table 1: Primers

YpdA/YpdB Primers	Tm (°C)	Sequence
ypdA KO_L	73.8	GTGCACGAAATATTCAACATGCTGCTGGCGGTCTTCGATCGGGCCGCGTTGTGTAGGCTGGAGCTGCTTC
ypdA KO_R	71.2	CCA GGC GGC GGA TAT GCA GCC CCT CGC CAT ACA ATA ACT TCA CGC GAT GACATATGAATATCCTCCTTAG
Test_ypdA KO_L	56.3	CTCAAAAACGGCCTGCTGGTC
Test_ypdA KO_R	56	ATG ACT TTC ACA ATA TCA CTC CGG C
ypdB KO_L	72.2	CAGGAACTGAGCTGGCTAATTAAAGAGCACAGCCAGATGGAGATTGTCGGCGTGTAGGCTGGAGCTGCTTC
ypdB KO_R	68.6	TTACAGATGCATTAACTGGCGGAATTCTTTAACTTTGCTACGGCTGACCG CATATGAATATCCTCCTTAG
Test_ypdB KO_L	56	GCCGGAGTGATATTGTGAAAGTCAT
Test_ypdB KO_R	56.7	AAT TGT TGA TCG GCG GGC AAG C
BtsS/BtsR Primers		**Note: BtsR was formerly annotated as YehT. BtsS was formerly YehU**
yehT KO_L	72.7	GGCCTGGCCGTTATCTTCCAGACGGATCTCCTGTAAATGCGCGAGGTTAAGTGTAGGCTGGAGCTGCTTC
yehT KO_R	69.4	AGGAGCAGAGCGATATTGAAATCGTTGGAGAGTGTTCAAACGCCGTAGAAGGCATATGAATATCCTCCTTAG
Test_yehT KO_L	57.9	CTGACCGGCACGGTTAAGCC
Test_yehT KO_R	57.2	AAA GTC TTA ATT GTC GAT GAT GAA CCG C
yehU_ KO_L	74.6	GGCCTGTTCGCTGTCGCGGCGGATCACCGCTTTAATGGTGTTAAGCGCGTGTAGGCTGGAGCTGCTTC
yehU_KO_R	68.8	GTGTGGTTTGCGGGTATGTACGATTTTAATCTGGTGTTGCTGCTGCTTCACATATGAATATCCTCCTTAG
Test_yehU KO_L	57	GATAAGCGTTCACATGTTCAATTTCGTC
Test_yehU KO_R	57.3	GCA AGA GTT CAA AGA AAG TTA AAC GCA AG
YhjX & YjiY Primers		
yhjX KO_F	79.16	CGGTTTATACCTGGAGTCTGTTTAACGGTGCGCTTTCTGCCAAGCTGGGGGGTGTAGGCTGGAGCTGCTTC
yhjX KO_R	71.87	GAAAGTCACATAGAATCCGCCAAACAGTGAAGCGATAATCCATATGAATATCCTCCTTAG
Test_yhjX KO_F	58.5	GAGATTTTTCCTTTTTATTACTGC
Test_yhjX KO_R	62.4	AACATTTTCTGCTCTGGCTG
yjiY KO_F	81.96	GTCGGGGGGAGCACGTCAGCGCCCTGTGGATCGTGGTCGCCTCTGTGTCGGTGTAGGCTGGAGCTGCTTC
yjiY KO_R	75.04	CAATCAGGAACAGAATACTCAGGCCTGCGTTGGTGTAGTTGTTCACAACGCATATGAATATCCTCCTTAG
Test_yjiY KO_F	59.9	TGCCAGGTTTTACTATGGATAC
Test_yjiY KO_R	60.7	AACCGTAGAAGATGATGCTGTA

Final Thoughts

I have enjoyed my time working with the YpdAB and BtsRS TCSs and I believe that there is much knowledge yet to be gained by investigating them further. As someone interested in cellular signaling processes, human health, and in mitigating the burden of pathogens, these systems have been exciting to work with and I look forward to seeing what new discoveries are yet to be found and seeing what new questions have yet to be asked. Coming to better understand these systems has the potential to further refine our view of bacterial TCS signaling cascades, demonstrating that they do sometimes work together in complex and at times unexpected ways. Continued study of these systems also has the potential to show a role for them in acid resistance: an important aspect of survival for pathogens such as UPEC. By coming to understand the regulation and function of TCS biology we have the potential to identify new targets for next generation antimicrobials that could potentially improve human health and save lives.

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