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Host Stress and Virulence Expression in Intestinal Pathogens: Development of Therapeutic Strategies Using Mice and *C. elegans*

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Abstract

The intestinal tract of a host exposed to extreme physiologic stress and modern medical intervention represents a relatively unexplored yet important area of infection research, given the frequency with which this site becomes colonized by highly pathogenic microorganisms that cause subsequent sepsis. Our laboratory has focused on the host tissue derived environmental cues that are released into the intestinal tract during extreme physiologic stress that induce the expression of virulence in colonizing pathogens with the goal of developing novel gut directed therapies that maintain host pathogen neutrality through the course of host stress.

Here we demonstrate that maintenance of phosphate sufficiency/abundance within the intestinal microenvironment may be considered as a universal strategy to prevent virulence activation across a broad range of pathogens that colonize the gut and cause sepsis, given that phosphate depletion occurs following stress and is a universal cue that activates the virulence of a wide variety of organisms. Using small animal models (*Caenorhabditis elegans* and mice) to create local phosphate depletion at sites of colonization of *Pseudomonas aeruginosa*, a common cause of lethal gut-derived sepsis, we demonstrate the importance of maintaining phosphate sufficiency to suppress the expression of a lethal phenotype during extreme physiologic stress. The molecular details and potential therapeutic implications are reviewed.

I. INTRODUCTION

Controlling infection in critically ill patients is an ever growing challenge as medical advances push the limits of human tolerance to physiologic stress, immune alterations, and injury. Solid organ and bone marrow transplantation, combined radiation and chemotherapy for cancer, total mechanical support of the heart and lungs, and even spaceflight- all impose a yet- to be defined array of selective pressures on the colonizing intestinal flora to remain viable and stable through the course of treatment [1–7]. A more complicating issue is the promiscuous use of powerful and multiple antibiotics- a practice that is virtually unavoidable during these extreme therapies [8, 9]. The recognition that the normal colonizing intestinal

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microbiota becomes rapidly replaced by multi-drug resistant and potentially virulent hospital pathogens presents a formidable challenge to monitor and understand the consequences of this abnormal carrier state [10–12].

In this chapter we will present the hypothesis that the gut of a critically ill host presents harsh and nutrient scarce environmental conditions that force colonizing hospital pathogens to respond with enhanced virulence. In extreme cases of physiologic stress imposed by medical intervention to treat disease and support life, intestinal pathogens are exposed to conflicting and paradoxical local cues released by host tissues indicating little precedent for survival. Short sighted microbes may then make the fundamental tradeoff to such environmental constraints by killing their host. In this report we posit that understanding the unique dimensionality of the process by which intestinal microbes survive under these harsh conditions can lead to environmental control strategies that can interdict in at very proximate points of microbial virulence activation. One such approach may be to maintain phosphate abundance, an important signal that can suppress microbial virulence activation, at precise sites of microbial colonization such as the distal intestinal tract mucus [13–15]. For example, under normal conditions, mucus is highly abundant in organic phosphate requiring that microbial phosphatases cleave organic forms to acquire phosphate. In order to successfully colonize the gut, microbes must adhere to and survive within the intestinal mucus layer where they obtain nutrients and avoid the sweeping action of peristalsis. Under conditions of physiologic stress however, intestinal mucus and phosphate rich phospholipids become rapidly depleted not only depriving colonizing microbes from a rich source of phosphate but also exposing the underlying epithelium as anchoring sites [13]. Thus by understanding the difficulties faced by bacteria under these circumstances and the means with which they respond, a unique opportunity to interdict in this process is offered as it potentially represents the most proximate point of pathogenesis. Here we present two small animal models that impose a degree of physiologic stress that can shift colonizing strains of intestinal *Pseudomonas aeruginosa* to express enhanced lethality in a manner that appears to be dependent on the local concentration of phosphate. The development of a mouse model that recreates physiologic stress imposed by surgical injury is highly suitable to develop therapies that can target the intestinal microenvironment [16]. The use of the *Caenorhabditis elegans* model provides a complementary system that is more ordered allowing for rigorous testing of the role of specific agents within the local microenvironment that can be manipulated to legislate molecular diplomacy between pathogen and host [14]. These models are ideally suited to provide the flexibility and fidelity needed to elucidate pathogenic mechanism of virulence activation of microbes in response to local environmental cues and to test therapeutic strategies aimed at local microenvironmental control to prevent virulence expression in the first place.

II. EXTREME PHYSIOLOGIC STRESS AND CRITICAL CARE THERAPY POSE DISORDER AND ADVERSITY TO THE INTESTINAL MICROBIOTA

From point of view of the intestinal microbiota, critical illness and its attendant advanced supportive therapy poses both disorder and adversity in a variety of ways (Fig. 1). In humans critical illness today, physiologic response systems are considered to be recently developed

and maladaptive since prior to advanced supportive therapy, the host would otherwise not survive. Disorders such as severe traumatic and burn injury, pharmacologic immunosuppression, solid organ and bone marrow transplantation, radiation therapy, etc, have highly destabilizing effects on the normal intestinal microbiota and pose difficulties for stable communities to persist. During critical illness multiple physiologic “hits” develop such as hypoxia and ischemia from low blood pressure and use of vasoactive agents, reperfusion metabolite production with the generation of reactive oxygen species, the need for repeated surgical intervention, placement of prosthetic materials etc. The content and function of the gastrointestinal tract is altered often precluding delivery of nutrients via this route. As such nutrients are delivered intravenously depriving the intestinal microbiota of its customary food. To prevent acid erosion of the stomach, powerful acid suppressing agents are used. As such there are an infinite number of local microenvironmental elements that are altered in the human intestinal tract that exert selective pressure on communities of microorganisms to cope in a contradictory or paradoxical manner. By contradictory we mean in a manner that would cause further injury to the host, which may not be in the pathogens best interest, at least in the short term.

Owing to the promiscuous use of antibiotics and the ubiquitous presence of highly pathogenic bacteria in hospital environments, most critically ill patients are intestinally colonized with *P. aeruginosa* [17–21]. Agents released into the gut during physiologic stress that activate *P. aeruginosa* to express enhanced virulence include interferon gamma (INF- γ) [22], adenosine [23], and opioids such as dynorphin [24]. Thus passage of a microbial pathogen through the gut of critically ill host can result in exposure to selective pressures (as soluble host derived agents and physico-chemical cues pH, phosphate etc) that change its behavior where it can spread endogenously (gut→blood, catheter, organ), contiguously (gut→lung), discontinuously (gut→ peritoneum), or extracorporeally (fecal→wound). Therefore a new paradigm of vulnerability of the critically ill to infection is not just chance exposure to an environmental pathogen, but transformation of the pathogen as a result of exposure to the host gut environment. Therefore along the lines of this hypothesis several questions remain: what are the local and host derived cues to which microbes are exposed during critical illness? How are they intercepted, processed and transduced by colonizing pathogens? What techniques can be used to identify them more precisely in the human intestinal tract during critical illness? Once identified, how can we reverse engineer the intestinal tract microenvironment using animal models such as *C. elegans* and mice in order to test strategies that will inhibit virulence transformation when microbes are exposed to the selective pressures of the gut during critical illness?

Investigators are finally beginning to recognize the importance of understanding the details of interkingdom signaling in the direction of host to pathogen. Some have termed the process by which microbes sense host tissue derived molecules as “telesensing” to reflect the concept that the molecular dialogue between host and pathogen does develop in the direction of host to pathogen and that the process itself might occur remotely [25, 26]. While the idea that host tissues signal microbes has been proposed by others [27–30], that this occurs in a context dependent manner relevant to physiologic stress is just beginning to be revealed [31–33]. While there may be many mechanisms by which this occurs including interkingdom gene exchange, investigators have focused on interkingdom quorum sensing,

since these systems are fairly well established in a variety of pathogens of interest [34]. For example we have discovered that *P. aeruginosa* recognizes the presence of INF- γ via specific binding to its outer membrane protein OprF, the ligation of which is sufficient and required for the activation of the core quorum sensing regulatory system RhlRI [22]. It has been recently shown that the quorum sensing regulator RhlR is required for *P. aeruginosa* to suppress the insulin-signaling DAF2/DAF16 pathway in *C. elegans* implicating innate immunity in this process [35]. This suggests that *P. aeruginosa* can recognize inflammation in its host via soluble INF γ and potentially suppress the immune response in order to avoid immune-mediated clearance. C4-HSL, a quorum sensing molecule secreted by *P. aeruginosa* in response to INF γ has been shown to modify the NFkB signaling system [36] providing yet another example of the back and forth aspect of the ongoing interkingdom molecular dialogue. Among the multiple genes in the quorum sensing regulon under RhlRI regulation in *P. aeruginosa* is the gene *lecA* that encodes the PA-IL protein, an adhesin that mediates adherence to and disruption of the intestinal epithelium [16, 37].

Intestinal ischemia and hypoxia are physiologic disturbances that invariably complicate the course of critically ill patients. Vasopressor use, direct vascular occlusion during surgery, and hemorrhage lead to episodes of intestinal hypoxia that can directly activate quorum sensing in *P. aeruginosa* resulting in the expression of the barrier disrupting protein PA-IL [23]. Interestingly *P. aeruginosa* can metabolize extracellularly secreted adenosine via adenosine deaminase to inosine which itself is capable of inducing the expression of the quorum sensing dependent virulence-related protein PA-IL. Extracellularly secreted adenosine by the intestine exposed to hypoxia is an important cytoprotectant that maintains tight junctional barrier function [38, 39]. The ability of *P. aeruginosa* to metabolize adenosine to inosine and deprive the epithelium of an important cytoprotectant while at the same time transducing its quorum sensing circuitry is a clever tactic to attach to the epithelium and gain entry into deeper tissues.

Analgesic opioids are invariably used in the care of the critically ill and themselves can activate quorum sensing in *P. aeruginosa* [24]. Endogenously generated opioids are also secreted into the intestine by immune cells such as neutrophils that produce and release opioids at precise sites of injury [40, 41]. The gut is often named “the second brain” owing to the presence of its dense neural network which often uses opioids as transmitter compounds [42–45]. We previously demonstrated that kappa opioid receptor ligand such as the endogenously produced peptide dynorphin or the chemically synthesized compound U-50,488 specifically activate the MvfR-PQS pathway in *P. aeruginosa* leading to the activation of multiple quorum sensing-regulated virulence factors [24]. We further demonstrated that dynorphin is released in significant quantities in the intestinal lumen during intestinal hypoxia and can be identified at specific sites of *P. aeruginosa* colonization where it colocalizes to cell cytoplasm of *P. aeruginosa* [24].

III. LOCAL INTESTINAL PHOSPHATE CONCENTRATION: A UNIVERSAL CUE TO SENSE HOST HEALTH STATUS DURING SURGICAL INJURY

The importance of phosphate to all living systems is obvious. As such it is not surprising that microbes have evolved various systems to recognize the extracellular concentration of phosphate and respond accordingly. Phosphate acquisition systems exist in virtually all microbes and in many cases regulate virulence expression. Yet whether phosphate depletion alone is sufficient to shift microbes to express a lethal phenotype remained unknown. From the standpoint of a colonizing microbe, especially one that is subjected to the harsh environment of the intestinal tract of a critically ill patient, the degree to which phosphate depletion exists and the degree to which it influences microbial phenotype behavior needs to be clarified. Serum phosphate depletion (hypophosphatemia) is an independent predictor of infection-related mortality among the critically ill patients when blood levels decrease below 1 mg/dL (0.3 mM) [46]. Critical illness and physiologic stress are known to redistribute body stores of phosphate via the circulating hormone phosphatonin [47, 48]. Stress mediated phosphatonin release, such as occurs following surgical, burn, and traumatic injury, causing an increase in intestinal phosphate absorption and increase phosphate excretion in the urine. Experimental models demonstrate severe serum phosphate depletion following physiologic stress [49]. Yet whether physiologic or surgical stress depletes phosphate at critical sites of microbial colonization, such as the intestinal tract mucus is unknown. To test this we subjected mice to surgical injury in the form a partial liver resection and discovered that intestinal mucus phosphate concentration decreased to levels that can activate quorum sensing dependent virulence expression in *P. aeruginosa* [13] (Fig. 2). Importantly neither serum nor luminal levels of phosphate decreased significantly. While injury induced mucus phosphate depletion could be prevented by oral loading of phosphate, intravenous phosphate loading did not restore mucus phosphate levels. When *P. aeruginosa* was introduced into the intestinal tract of mice following surgical injury, mortality was dependent on the local concentration of phosphate. Surgically injured mice intestinally inoculated with *P. aeruginosa* had a high mortality rate compared to identically treated mice orally loaded with inorganic phosphate [13]. Retrieval of strains from mice subjected to surgical injury and *P. aeruginosa* demonstrated a 32 fold increase in the expression of the phosphate acquisition protein PstS, whereas in mice supplemented with oral phosphate, PstS expression was low. These findings were prominent in the intestinal mucus but not when strains from the lumen were analyzed (where phosphate concentration is normal) suggesting that the local level of phosphate at the precise site of microbial colonization (i.e mucus) may be the determinant of phosphate related virulence activation. Yet whether phosphate depletion alone was sufficient to explain the shift of *P. aeruginosa* to a lethal phenotype in mice following surgical injury required a more reductionist and ordered host system.

IV. USE OF *C. ELEGANS* TO DETERMINE THE MECHANISMS OF PHOSPHATE DEPENDENT LETHALITY OF *P. AERUGINOSA*

While the mouse model of partial liver resection developed in our laboratory results in intestinal mucus phosphate depletion [13], it is likely to also affect multiple perturbations of several other environmental and physiologic elements within the intestinal tract that might

affect microbial virulence. For example, experimental liver resection is known to enhance the secretion of bile acids up to 24 fold [50, 51] which can alter epithelial barrier function as well as shift bacterial phenotype [52–54]. Intestinal blood flow is also affected by this type of surgery and therefore mucosal pH, oxygen tension, iron availability, etc is also likely to be significantly altered.

To test the independent effect of phosphate depletion on *P. aeruginosa* lethality, we developed a *C. elegans* model in which phosphate depletion of microbial lawns could be created upon which the nematodes feed to determine the specific level at which phosphate concentration affects microbial killing of its host via the digestive tube. In order to do this, we modified the *C. elegans* model by including two major perturbations to simulate the mouse model: 1.) *P. aeruginosa* lawns were grown on nematode growth media without the addition of phosphate (NGM↓Pi), and 2.) nematodes were pre-starved to empty their intestinal tube [14]. Under these conditions, *C. elegans* feeding on *P. aeruginosa* PAO1 lawns died at a high rate of 60–100% at 24–48 hours. No mortality was found in worms feeding on the lawns of PAO1 grown on NGM containing 25 mM potassium phosphate buffer, pH 6.0 (NGM↑Pi), or feeding on *E. coli* OP50 under either conditions (Fig. 3). We next performed reiterative experiments to transcriptionally profile *P. aeruginosa* grown under high and low phosphate conditions as high density lawns. Results demonstrated involvement of three global virulence subsystems: low phosphate signaling and acquisition, iron acquisition, and the MvfR-PQS quorum sensing subsystem. The functional role of the PhoB transcriptional regulator of PHO regulon in the activation of MvfR-PQS pathway, and the role of PQS to bind excess of Fe³⁺ delivered by the siderophore pyoverdine (iron acquisition subsystem) with the formation of [PQS-Fe³⁺] complex were determined and found to play a critical role on the induction of a lethal phenotype in *P. aeruginosa* under phosphate limitation (Fig. 4). Death of nematodes on low phosphate lawns of PAO1 was associated with red colored material appearing in the pharynx and digestive tube. Given that the [PQS-Fe³⁺] complex is a red colored complex, we hypothesized that the appearance of the red material in the worm digestive tube was likely to be a PQS-Fe³⁺ complex. To show this we performed reiterative studies with knockout mutants of either *mvfR* (encoding transcriptional regulator involved in PQS biosynthesis), *pqsA* (proximal gene in *pqsABCDE* operon involved in PQS biosynthesis), or *pvdD* involved in pyoverdine biosynthesis. Results demonstrated that intact *mvfR*, *pqsA*, and *pvdD* are required to produce the red colored material that was found the digestive tube of *C. elegans* feeding on low phosphate PAO1 mutant lawns.

To confirm the direct toxicity of the [PQS-Fe³⁺] complex, worms were allowed to feed on artificial lawns containing this complex with the addition of rhamnolipids. Rhamnolipids, a *P. aeruginosa* “wetting agent” were added to solubilize PQS, a highly hydrophobic compound that otherwise precipitates in aqueous solution. The [PQS-Fe³⁺-rhamnolipids] complex was solubilized in water and mixed with non-viable *E. coli* OP50 debris to allow the complex to be absorbed by the solid particles thereby preventing it from becoming absorbed in the agar. Finally, the whole mixture was pored onto the plain solidified agar as an “artificial” lawn onto which worms were transferred. The triple [PQS-Fe³⁺-rhamnolipids] complex was found to be highly lethal for worms whereas the [PQS-rhamnolipids] or [PQS-Fe³⁺] complexes were less toxic effect (Fig. 5). Thus the *C. elegans* model was extremely useful in reverse engineering the intestinal microenvironment to expose *P. aeruginosa* to

depleted phosphate conditions and thereby allowing for the elucidation of the molecular mechanisms of its transition to a lethal phenotype within the digestive tube. We were therefore able to prove that phosphate alone is sufficient to shift *P. aeruginosa* to a lethal phenotype within the digestive tube of a small animal.

V. FIDELITY BETWEEN MOUSE AND *C. ELEGANS* MODELS ON INTESTINAL *P. AERUGINOSA* PATHOGENESIS

Although *C. elegans* has been enormously useful in identifying pathways of virulence across various pathogens, it is frequently used to perform high throughput mutant screens [55–59]. Reports of high fidelity with this approach have been documented in both mice and plants underscoring the usefulness and relevance of the model [55, 60–62]. Our work with *C. elegans* that led us to discover that soluble virulence products (i.e [PQS-Fe³⁺-rhamnolipids]) can be toxic to *C. elegans* when ingested into the digestive was also able to be verified in mice for its clinical relevance. When we injected this mixture into the mouse cecum we observed that it caused superficial epithelial cell disruption, inflammation, and epithelial apoptosis. Importantly mice developed signs of severe sepsis such as lethargy, ruffled fur, and shivering with significant mortality. Thus a novel mechanism by which exoproducts of intestinal *P. aeruginosa* can cause occult lethal sepsis was discovered by reductionist experiments with *C. elegans*. Neither PQS nor rhamnolipids alone induced sepsis or mortality in mice.

P. aeruginosa is one of the most common pathogens associated with lethal sepsis syndrome among the critically ill [31, 63, 64]. *P. aeruginosa* is present in the feces of up to 75% of critically ill patients and molecular identification studies have coupled subsequent infections with *P. aeruginosa* to its primary site of colonization- the gastrointestinal tract. In many cases *P. aeruginosa* can cause lethal sepsis from within the gastrointestinal tract that completely eludes clinical detection as blood cultures remain sterile throughout the course of sepsis or are sterilized by antibiotics [65]. Previous work in our lab demonstrated this later concept by demonstrating that an important barrier disrupting protein in *P. aeruginosa*, the PA-IL lectin is *in vivo* expressed in the intestinal tract during surgical injury in response to local host tissue derived cues [22, 24]. The PA-I lectin can induce a tight junctional permeability defect to potent cytotoxins such as elastase and exotoxin A via alterations in key cytoskeletal proteins such as occluding and actin [16]. Using purified proteins as well and mutant strains, we demonstrated intestinal origin lethal sepsis can result from permeation of intestinal exotoxin A caused by tight junctional barrier defect caused by PA-IL. Taken together with our previous observations, permeation of highly toxic compounds across the epithelial barrier secreted by *P. aeruginosa* in response local environmental cues released during surgical injury can cause lethal sepsis that may be difficult to detect clinically. Furthermore this mechanism of pathogenesis suggests that interventions that maintain an environment that is neutral to the pathogen in terms of virulence activation could prevent infections that are gut-derived among the critically ill. This approach would depart from current attempts to control the gut microbiota during critical illness that use digestive tract decontamination with antibiotics or probiotic therapy neither of which has proven satisfactory. Maintaining phosphate abundance at sites of local microbial colonization with compounds that can be

orally delivered to the distal intestine and remain durable can be tested in *C. elegans* using high throughput assays. Screening large libraries of compounds that interfere with microbial virulence activation in response to known host tissue derived signaling compounds could also be tested in *C. elegans* and verified in mouse models. Our work in this area with *P. aeruginosa* suggests that the fidelity between *C. elegans* and mouse models to screen for inhibitory compounds is suitable to create a structure function pipeline of discovery to identify compounds that will function well within the digestive system of animals.

VI. CONCLUSION

The recognition that information processing of environmental cues by microbes is a major mechanism of virulence activation that appears to play a key role by which they cause lethal sepsis within the gut offers various opportunities for mechanistic and therapeutic based studies. The approach of preserving bacterial viability and growth in the intestine by developing non-microbiocidal compounds that interdict in host to pathogen communication offers the most promise as a long term ecological strategy. Phosphate may be one agent that can be manipulated in this process since phosphate signaling via PstS-PhoB and analogous systems is highly conserved across a wide variety of clinically problematic gram-negative and gram-positive bacteria. However as it is well recognized that the intestinal tract during extreme physiologic stress is a hostile, nutrient scarce, and ecologically disturbed microenvironment, elucidation of the multiple *in vivo* cues that trigger virulence in problematic pathogens could provide new knowledge on emergent properties in bacteria that are able to persist in this environment. Use of small animal models such as *C. elegans* subjected to starvation stress and mice subjected to surgical injury could lead to novel therapeutic strategies aimed at maintaining ecological neutrality (i.e. phosphate abundance) with the goal of suppressing virulence expression at the outset.

ABBREVIATIONS

NGM	Nematode Growth Medium
PQS	Pseudomonas quinolone signal
PA-IL	PA-I lectin protein of <i>P. aeruginosa</i>
MvfR	multiple virulence factor regulator

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Fig. 1.

The potential role of aggregated disordering agents on the intestinal microbiota that accrue from the effects and treatment modalities of human critical illness.

Fig. 2. Mouse model of a surgical (30%) liver resection (hepatectomy) with simultaneous intestinal exposure to *P. aeruginosa* that leads to acute phosphate depletion in the distal (ileum, cecum) intestinal mucus layer.

Fig. 3.
C. elegans model of phosphate limitation.

Fig. 4.

Discovery platform of the *C. elegans*-*P. aeruginosa* model. A. Genes are identified from whole genome transcriptional profiling. B. Post translational studies are conducted by measuring secreted compounds directly (MS, fluorescence) or using protein fusion reporter constructs. C. Selected mutants of identified systems are screened for their killing effect on *C. elegans*. D. Data are then reconstructed, hypotheses developed, and then re-interrogated in the *C. elegans* model and in mice.

Fig. 5. Artificial lawns containing [PQS-Fe³⁺-rhamnolipid] complex. **A.** Creation of artificial lawns. **B.** Accumulation of the red colored complex inside the intestinal tube of worms (arrow). **C.** Mortality in worms feeding on artificial lawns.