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Dynorphin Activates Quorum Sensing Quinolone Signaling in Pseudomonas aeruginosa

Olga Zaborina1, Francois Lepine2, Gaoping Xiao3, Vesta Valuckaite4, Yimei Chen5, Terry Li6, Mae Ciancio4, Alex Zaborina1, Elaine Petroff2, Jerrold R. Turner7, Laurence G. Rahme3, Eugene Chang4, John C. Alverdy1*

1 Department of Surgery, Pritzker School of Medicine, University of Chicago, Chicago, Illinois, United States of America, 2 Institut National de la Recherche Scientifique (INRS)–Institut Armand-Frappier, Laval, Quebec, Canada, 3 Department of Surgery, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts, United States of America, 4 Department of Medicine, Pritzker School of Medicine, University of Chicago, Chicago, Illinois, United States of America, 5 Department of Biochemistry and Molecular Biology, Pritzker School of Medicine, University of Chicago, Chicago, Illinois, United States of America, 6 Department of Immunohistochemistry, Pritzker School of Medicine, University of Chicago, Chicago, Illinois, United States of America, 7 Department of Pathology, Pritzker School of Medicine, University of Chicago, Chicago, Illinois, United States of America

There is now substantial evidence that compounds released during host stress directly activate the virulence of certain opportunistic pathogens. Here, we considered that endogenous opioids might function as such compounds, given that they are among the first signals to be released at multiple tissue sites during host stress. We tested the ability of various opioid compounds to enhance the virulence of Pseudomonas aeruginosa using pyocyanin production as a biological readout, and demonstrated enhanced virulence when P. aeruginosa was exposed to synthetic (U-50,488) and endogenous (dynorphin) κ-agonists. Using various mutants and reporter strains of P. aeruginosa, we identified involvement of key elements of the quorum sensing circuitry such as the global transcriptional regulator MvfR and the quorum sensing-related quinolone signaling molecules PQS, HHQ, and HQNO that respond to κ-opioids. The in vivo significance of κ-opioid signaling of P. aeruginosa was demonstrated in mice by showing that dynorphin is released from the intestinal mucosa following ischemia/reperfusion injury, activates quinolone signaling in P. aeruginosa, and enhances the virulence of P. aeruginosa against Lactobacillus spp. and Caenorhabditis elegans. Taken together, these data demonstrate that P. aeruginosa can intercept opioid compounds released during host stress and integrate them into core elements of quorum sensing circuitry leading to enhanced virulence.

Introduction

It has been suggested for microbial pathogens that colonize the mucosal surface of a healthy host that symbiosis can be viewed as a form of molecular détente, a settled negotiation that is sustained by an ongoing chemical dialogue between the host and its flora [1]. Even for an opportunistic pathogen, virulence expression against its host presents a fundamental tradeoff in that it will provoke immune retaliation and deplete the host of critical resources, and, as such, bacteria are constantly assessing the costs versus benefits of expressing virulence. Although bacteria use complex systems of communication like the quorum sensing signaling (QS) system to collect, process, and share information about the chemical composition of their environment [2], whether such events are influenced by specific host-derived signals that indicate a major change in host health status is less well defined.

Our laboratory has been interested in host-derived bacterial signaling compounds that are proximate causes of microbial virulence activation during physiologic stress. To date, several host-derived bacterial signaling compounds have been identified that include adaptive elements of the immune system such as interferon γ [3], tumor necrosis factor α [4], and interleukin-1 [5], as well as innate elements including adenosine [6], epinephrine [7–10], and antimicrobial peptides [11–13]. While in vitro exposure to various host compounds can activate the virulence of bacteria, much remains to be learned about how these compounds are collected, processed, and transduced within the various virulence regulatory systems of bacteria.

One of the best studied systems of virulence regulation in bacteria is the QS system. The QS system functions via autoinducer molecules that are released and taken up by bacteria to provide a cell–cell communication network whereby complex assemblage behavior can be carried out by large populations of bacteria responding to local concentrations of QS molecules [2]. In some cases, host-derived bacterial signaling molecules such as epinephrine have been shown to act as a surrogate QS autoinducer molecule [8], activating various virulence genes in intestinal bacteria such as Escherichia coli. In other cases, the QS system is activated by the binding of host-derived bacterial signaling molecules to specific membrane receptors on the bacteria, such as when...
interferon \( \gamma \) binds to the OprF outer membrane protein in *Pseudomonas aeruginosa* [3].

We considered that opioids might function as host-derived bacterial signaling molecules given that endogenous opioids are broadly distributed within the richly innervated intestinal mucosa and exert multiple effects during stress in neuronal, immune, and intestinal epithelial cells [14–17]. The intestinal tract represents a unique intersection of opioids and bacteria given the high abundance of peripheral neurons, immune cells, and bacteria in this site. Three main families of opioids have been identified based on their affinity to \( \delta \)-, \( \mu \)-, and \( \kappa \)-opioid receptors [14] that include the endogenous opioids \( \beta \)-endorphin, enkephalin, and dynorphin [18–20]. Following stress, endogenous opioids have been shown to act as paracrine and autocrine signals with high levels of functional redundancy and pleiotropy [21]. These observations, coupled with the findings that neutrophils themselves can synthesize and release opioids at sites of inflammation, strongly suggest that bacteria are exposed to opioids during the course of infection.

We have been interested in the mechanism by which the human opportunistic pathogen *P. aeruginosa* is activated to express a virulent phenotype during stress. We have previously shown in mice that stress results in the release of soluble compounds into the intestinal lumen that directly activate the virulence of *P. aeruginosa* to disrupt the intestinal epithelial barrier [22]. Given the abundance of neurons and immune cells in the gut that could produce opioids, we exposed strains of *P. aeruginosa* to various opioids with specificity to \( \mu \)-, \( \lambda \)-, and \( \kappa \)-opioid receptors and used pyocyanin (PCN) production as a biologic readout for virulence expression. Results demonstrated that only the \( \kappa \)-opioid receptor agonist U-50,488 induced PCN production in a dose-dependent manner. Next, we examined the effect of dynorphin, a naturally occurring \( \kappa \)-opioid peptide known to be present in the mammalian intestine, on its ability to produce PCN in *P. aeruginosa*, and found that dynorphin potently induced PCN production. In an in vivo stress model in mice, we demonstrated that dynorphin is released into the intestinal lumen and binds to desquamated epithelia and intestinal *P. aeruginosa*. Dynorphin was found to penetrate the bacterial membrane and directly induce the expression of the multiple virulence factor regulator (MvfR)–regulated operon *pqsABCDE*, resulting in enhanced production of three known intercellular QS-related signals, 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), 4-hydroxy-2-heptylquinoline (HHQ), and *Pseudomonas* quinolone signal (5,4-dihydroxy-2-heptylquinoline (PQS)) [23]. Exposure of *P. aeruginosa* strain PAO1 to \( \kappa \)-opioid receptor agonists U-50,488 and dynorphin resulted in enhanced virulence as judged by suppressed growth of the probiotic microorganisms *Lactobacillus* spp. and the nematode *Caenorhabditis elegans*. Taken together, these studies provide novel insight into the mechanism by which *P. aeruginosa* is activated to express virulence in response to host stress by processing the opioid peptide dynorphin into its QS circuitry.

**Results**

**U-50,488 Stimulates *P. aeruginosa* PAO1 to Produce Pyocyanin**

Preliminary work in our laboratory demonstrated that exposure of *P. aeruginosa* to filtered intestinal contents from stressed mice induced an intensely green color (unpublished observation), suggesting that the blue-green pigment PCN, a known quorum sensing–dependent virulence factor [24], was produced by soluble compounds released into the intestinal tract during stress. In order to determine whether opioid compounds might be among the factors responsible for bacterial virulence activation during stress, we exposed *P. aeruginosa* PAO1 to \( \mu \)-, \( \kappa \)-, and \( \delta \)-opioid receptor agonists, and examined bacteria for a change in color and PCN production. Studies were performed using morphine, a predominately \( \mu \)-opioid agonist previously shown to be synthesized in animals [25,26], U-50,488, a specific synthetic \( \kappa \)-opioid, and BW373U86, a specific synthetic \( \delta \)-opioid agonist [14]. Figure 1A shows that \( \kappa \)-opioid U-50,488 induced an intensely bright green color in *P. aeruginosa* PAO1 that correlated to an increase in PCN production in a dose-dependent manner (Figure 1B). The \( \delta \)-opioid BW373U86, on the other hand, had an inhibitory effect on PCN production (Figure 1C), whereas exposure to the \( \mu \)-agonist morphine resulted in a bell-shaped type dose response curve for PCN (Figure 1D). Although exposure to morphine did not increase PCN to the same degree as with U-50,488 (~40% versus 300%), at lower cell densities when baseline levels of PCN were negligible, the effect of morphine appeared to be pronounced at the 50-\( \mu \)M dose (~600%) (Figure 1E). None of the opioids tested resulted in significant changes in the growth of *P. aeruginosa* (Figure 1F–1H).

Since PCN production is a quorum sensing–dependent virulence factor and is produced at high bacterial cell densities, we analyzed the effect of U-50,488 during bacterial growth. U-50,488 induced PCN production at earlier cell density without affecting bacterial growth, suggesting a regulatory shift in the quorum sensing circuitry of *P. aeruginosa* (Figure 1).
Dynorphin Induces P. aeruginosa Virulence
Production of PCN in Response to U-50,488 Requires an Intact QS System: Role of Proximal QS Regulatory Protein MvfR in Enhanced PCN Production in Response to U-50,488

Figure 2A outlines the critical pathways within the QS system that are involved in the regulation of PCN production. To verify that κ-opioid mediated activation of PCN production requires an intact QS system, *P. aeruginosa* PAO1 mutants defective in key transcriptional regulators RhlR, LasR, GacA, and MvfR, as well as the autoinducer synthetases RhlI and LasI, were exposed to U-50,488 at concentrations of up to 1 mM. All transcriptional regulator mutants failed to produce PCN both in the presence and absence of U-50,488, suggesting that an intact QS system is necessary for the κ-opioid–mediated effect (unpublished data). PCN production was partially restored in ΔRhlI and ΔLasI mutants by adding exogenous C4-HSL (Figure 2B). That the addition of C4-HSL had a minimal effect in the RhlI mutant can be explained by competitive binding of 3-oxo-C12-HSL to RhlR in the absence of C4-HSL. In the double mutant ΔLasIΔRhlI, PCN production was increased in response to C4-HSL, possibly as a result of the absence 3-oxo-C12-HSL. In contrast to C4-HSL, the κ-opioid agonist U-50,488 failed to restore PCN production under the same conditions, suggesting that κ-agonists cannot function as surrogate QS molecules. We next focused on two key proximal QS regulatory proteins, MvfR and GacA,
which are known to be critically important in PCN biosynthesis. The mutants were complemented with their respective genes, mvfR and gacA. While both complemented mutants produced PCN, responsiveness to the δ-agonist U-50,488 was only observed in ΔmvfR/mvfR (Figure 2C). Dynamic tracking of PCN production in the ΔmvfR/mvfR during growth again demonstrated enhanced PCN production in response to the δ-agonist (Figure 2D). An inhibitory effect of U-50,488 on PCN production was found in the complemented ΔGacA mutant (Figure 2C). The mechanism for this paradoxical effect is unknown. It is possible that complex interactions between the GacA and MvfR regulons that develop in the presence of high copies of GacA in the complemented mutant and in MvfR when activated by U-50,488 produce this dampening effect.

**Exposure of *P. aeruginosa* PAO1 to U-50,488 Results in Enhanced Expression of *pqsABCDE*, Production of HQNO, HHQ, and PQS, and PA-I Lectin Expression**

Because MvfR directs the transcription of the *pqsABCDE* operon [27], which is responsible for the biosynthesis of 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) as well as the direct precursor of the *Pseudomonas* quinolone signal HHQ [23], we exposed PAO1 to U-50,488 and examined the effect of U-50,488 on the expression of *pqsABCDE* and the production of HQNO, HHQ, and PQS. The expression of *pqsABCDE* was examined by measuring β-galactosidase activity in strain PAO1 harboring the pGX5 plasmid containing pgaA'–lacZ construction [28]. Figure 3A shows that exposure of PAO1 to U-50,488 resulted in enhanced expression of *pqsABCDE*. Next, we examined the effect of U-50,488 on mvfR expression in strain PAO1 harboring the pGX1 containing the mvfR'–lacZ fusion gene [29] and found that U-50,488 had no effect on mvfR expression (Figure 3B). The concentrations of PQS, HHQ, and HQNO were found to be elevated in PAO1 exposed to U-50,488 (Figure 3C). No differences were observed in the production of other important QS molecules C4-HSL and 3-oxo-C12-HSL in PAO1 grown in the presence or absence of U-50,488 (unpublished data). It has been recently reported that exposure of *P. aeruginosa* to PQS significantly increases PA-I lectin (PA-IL) expression [30]. Since U-50,488 enhanced PQS biosynthesis, we considered it might also stimulate PA-IL expression. PA-IL expression was dynamically tracked in response to U-50,488 using the green fluorescent PA-IL reporter strain 27853/PLL-EGFP previously constructed in our laboratory [31]. Marked differences in fluorescence were observed in this strain during growth in the absence and presence of U-50,488 (Figure 3D). Results were confirmed in strain PAO1 by real-time PCR (Figure 3E), demonstrating the increased expression of the *locA* gene encoding PA-IL following exposure to U-50,488. Expression of the housekeeping gene glnA encoding citrate synthase was analyzed under the same conditions, and no effect of U-50,488 on glnA expression was observed.

**The Naturally Occurring δ-Opioid Peptide Dynorphin Enhances the Expression of *pqsABCDE*, Leading to Increased Production of HQNO, HHQ, and PQS, the Expression of *phzA1-G1*, and Enhanced Biosynthesis of PCN**

Having established that opioid-induced PCN production in *P. aeruginosa* is specific to δ-receptor agonists, we next sought to determine whether naturally occurring endogenous δ-agonists could induce PCN production in *P. aeruginosa*. Among endogenous opioids, only dynorphin has been shown to be specific to the δ-receptor [32]. Therefore, we exposed PAO1 to varying concentrations of dynorphin A (1–17) (Sigma) and found a dose-dependent effect of dynorphin on PCN production (Figure 4A). We next determined if dynorphin increased the expression of *mvfR*, *pqsABCDE*, and *phzA1-G1*, key components involved in PCN regulation. The fusion constructs mvfR'–lacZ on pGX1 [23], pgaA'–lacZ on pGX5 [28], and phzABC-lacZ on MW303 [33] were introduced into strain PAO1. Similar to U-50,488, dynorphin did not increase MvfR expression in PAO1/mvfR'–lacZ (unpublished data); however, dynorphin increased β-galactosidase activity in both PAO1/mvfR'–lacZ (Figure 4B) and PAO1/pqsA'–lacZ (Figure 4C). When PAO1/pqsA'–lacZ was exposed to both dynorphin and PQS, β-galactosidase activity was increased above that observed with either dynorphin or PQS alone (Figure 4D), suggesting a synergistic effect of dynorphin and PQS on *pqsABCDE* expression.

Next, we determined if dynorphin increased *pqsABCDE* expression in the absence of PQS. We used a PAO1 derivative *pqsC* knockout mutant, strain MP603 [34]. Strain MP603/pqsA'–lacZ displayed only a baseline level of β-galactosidase activity both in the presence and absence of dynorphin; however, β-galactosidase activity was significantly increased in response to the combination of dynorphin and PQS compared to PQS alone (Figure 4E). The synergistic effect on *pqsABCDE* expression in strain MP603 was dependent on the relative concentrations of dynorphin and PQS. For example, no synergy was observed when the PQS concentration exceeded that of dynorphin, and similarly, an inhibitory effect was observed when the dynorphin concentration (>10-fold) exceeded that of PQS (unpublished data). Finally, similar to U-50,488, dynorphin increased HQNO, HHQ, and PQS production in PAO1 (Figure 4F).

**Dynorphin Accumulates in Intestinal Tissues during Stress, Is Released into the Intestinal Lumen, and Is Transferred to Bacteria within the Intestinal Lumen**

We hypothesized that bacteria might be exposed to dynorphin in vivo in the intestinal tract under clinically relevant pathophysiological conditions [35]. To test this, we exposed the mouse intestine to two conditions: 1) 30 min of ischemia followed by 30 min of reperfusion stress, and 2) ischemia/reperfusion (I/R) stress coupled with luminal inoculation with *P. aeruginosa* (I/R + Pa). Figure 5A–5C shows 4-μm intestinal sections isolated from (A) control, (B) I/R, and (C) I/R + Pa mice, and stained with hematoxylin and eosin. The black arrows on Figure 5B and 5C show desquamated epithelium that is a common feature of this injury. Figure 5D–5G shows immunohistochemical staining of intestinal segments for dynorphin in control samples (Figure 5D and 5G), dynorphin was found to be scarcely localized to the crypts (Figure 5D, red arrow), whereas following I/R injury, dynorphin was found to be abundantly present on the villus tips and within the intestinal lumen (Figure 5E and 5H, red arrows), a finding that appeared to be enhanced in the presence of luminal *P. aeruginosa* (Figure 5F and 5I, red arrows). Examination of bacteria within the intestinal lumen and on the epithelial surface demonstrated positive dynor-
Dynorphin staining bacteria at various sites, including bacteria attached to intestinal epithelial cells (Figure 5J–5M).

To determine the concentration of dynorphin in the luminal contents of intestinal segments subjected to I/R and I/R + Pa, 10-cm segments were flushed with 2 ml of phosphate buffered saline (PBS) containing protease inhibitor cocktail (Roche), and samples assayed using competitive enzyme-linked immunosorbent assay (ELISA). Figure 5N shows a significant increase in luminal dynorphin in mice subjected to I/R injury that was further increased when I/R was coupled

**Figure 3.** U-50,488 Induces *pqsABCDE* Expression, Biosynthesis of HQNO, HHQ, and PQS, and Stimulates PA-IL Expression

Error bars, mean ± SD.
(A) Effect of U-50,488, 200 μM and PQS, 100 μM on *pqsA*-lacZ expression in *P. aeruginosa* strain PAO1/pGX5 following 5 h of incubation.
(B) Effect of U-50,488, 200 μM and PQS, 100 μM on *mvfR*-lacZ expression in *P. aeruginosa* strain PAO1/pGX1 following 5 h of incubation.
(C) Effect of U-50,488, 200 μM on HQNO, HHQ, and PQS production by *P. aeruginosa* PAO1. * p < 0.01.
(D) Dynamic tracking of PA-IL expression using PA-IL reporter strain *P. aeruginosa* 27853/PLL-EGFP.
(E) Real-time PCR of *lecA* encoding PA-IL and the housekeeping gene *gltA* encoding citrate synthase in *P. aeruginosa* PAO1 grown to OD600nm = 3.0 in the presence of 200 μM of U-50,488. The graph was made based on the Ct levels for *gltA*, 20.26 ± 0.81 (control) versus 20.78 ± 0.26 (U-50,488); and for *lecA*, 29.53 ± 0.43 (control) versus 27.42 ± 0.97 (U-50,488). Ct levels for *lecA* blank control (no template) were ~ 40.

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Dynorphin Induces *P. aeruginosa* Virulence
with luminal inoculation with *P. aeruginosa* (I/R + Pa). To define the putative role of dynorphin on PCN production in vivo, PAO1 was exposed to filtered \(0.22 \mu m\) luminal contents from each group of mice, and PCN production determined. Exposure of PAO1 to luminal flushings from intestinal segments of the various groups of mice demonstrated a significant correlation between dynorphin concentration in the luminal samples and its ability to induce PCN production \((R = 0.7987, \text{Figure 5O})\). Immunodepletion of dynorphin in samples using rabbit polyclonal anti-dynorphin antibody attenuated the ability of samples to induce PCN production in PAO1 (Figure 5P).

**Dynorphin Binds to *P. aeruginosa* In Vitro and Enters the Bacterial Cytoplasm**

To confirm that dynorphin can bind to bacteria, we performed in vitro staining of *P. aeruginosa* in the presence of dynorphin. Dynorphin \((100 \mu M)\) was added to *P. aeruginosa* at the early log phase, and incubated for 1 h. Cells were collected, washed, and fixed on slide. Dynorphin was detected by immunostaining using anti-dynorphin pAB. Figures 6A and 6B show negative dynorphin staining in the negative controls when cells were cultivated without dynorphin (A), and when cells were cultivated with dynorphin but primary antibodies were omitted and rabbit serum was used instead (B). Figure 6C demonstrates positive dynorphin staining in cells cultivated with dynorphin followed by treatment with anti-dynorphin antibody. Structurally, dynorphin is similar to other cell-penetrating peptides in that its high content of basic and hydrophobic amino acid residues facilitates its penetration through mammalian cell membranes [36]. Therefore, by using immunogold electron microscopy, we determined the ability of dynorphin to traverse the bacterial cell membrane.
Figure 5. In Vivo Production of Dynorphin in the Mouse Intestine during I/R

(A–C) Histology of small intestine from (A) control mice demonstrating intact mucosal epithelium, and (B) I/R and (C) I/R + Pa mice showing disruption of mucosal epithelium with desquamated epithelial cells inside the intestinal lumen (black arrows).
plasma membrane and enter the bacterial cell interior. Figure 6D shows an image of *P. aeruginosa* PAO1 cells incubated with dynorphin. Localization of *P. aeruginosa* PAO1 cells was identified by 10-nm gold particles (black arrows), which were found predominantly in the bacterial cytosol fraction close to the inner membrane.

**κ-Opioid Receptor Agonists U-50,488 and Dynorphin Enhance the Virulence of *P. aeruginosa* PAO1 against *Lactobacillus* and *C. elegans***

To determine the clinical relevance of the above findings, we examined the ability of κ-opioid receptor agonists to shift the virulence of *P. aeruginosa* against the nematode *C. elegans*.

**Figure 6. Dynorphin Binds to *P. aeruginosa* In Vitro, and Enters the Bacterial Cell Cytoplasm**

(A–C) Binding of dynorphin to *P. aeruginosa*; (A) negative control demonstrating no dynorphin staining when cells were not incubated with dynorphin; (B) negative control demonstrating no dynorphin staining when cells were incubated with dynorphin but primary anti-dynorphin antibodies were omitted from staining procedure; and (C) positive staining (brown color) of *P. aeruginosa* incubated with dynorphin followed by whole procedure of immunostaining.

(D) Immunoelectron microscopy of *P. aeruginosa* PAO1 cells incubated with dynorphin, 100 μM. Black arrows show 10-nm gold spots indicating the presence of dynorphin.

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In addition, we determined whether exposure *P. aeruginosa* to κ-opioid receptor agonists could affect the growth of the cytoprotective probiotic organisms *Lactobacillus plantarum* and *Lactobacillus rhamnosus* [37–40]. Media from *P. aeruginosa* PAO1 grown in the presence of U-50,488 suppressed the growth of *L. plantarum* and *L. rhamnosus* GG (Figure 7A and 7B), whereas U-50,488 alone had no effect (unpublished data). Similarly, media from *P. aeruginosa* PAO1 grown in the presence of dynorphin suppressed the growth of *Lactobacillus*, whereas dynorphin alone had no effect (Figure 7C and 7D). Conditioned media from the PAO1 ΔMvfR mutant grown in the presence or absence of dynorphin did not affect the growth of *Lactobacillus* spp., suggesting that the κ-mediated effect is regulated via MvfR (Figure 7E). *C. elegans* feeding on lawns of PAO1 exposed to U-50,488 or dynorphin demonstrated suppressed production of new progeny, an indicator of enhanced virulence (Figure 7F and 7G). In this assay, the PAO1 ΔMvfR mutant was observed to be significantly less virulent compared to the wild-type PAO1 (Figure 7F), and its virulence was not enhanced in the presence of κ-agonists (Figure 7F and 7G).

**Discussion**

In animals exposed to physiologic or traumatic stress, subsequent bacterial challenge has been shown to result in increased mortality [10,31] in association with impaired immune function and bacterial clearance [17,41,42]. Data from the present study add to the small but expanding body of data showing that soluble compounds released by the host during stress and immune activation can directly interact with pathways of bacterial virulence regulation in a highly...
Dynorphin Induces *P. aeruginosa* Virulence

Figure 8. Proposed Activation and Effectors Pathways of *P. aeruginosa* in Response to Host Stress (Intestinal I/R Injury)

(1) Dynorphin is released by intestinal tissues and accumulates in the lumen during ischemia/reperfusion and penetrates the plasma membrane of *P. aeruginosa* (dark green arrows).

(2) Dynorphin synergizes with PQS via MvfR to increase the transcription of *pqsABCDE* leading to the production of HAQs, including HQNO and HHQ.

(3) Increased HQNO production suppresses the growth of *Lactobacillus* spp., rendering the intestinal epithelium more vulnerable to invasion and the action of cytotoxins of *P. aeruginosa* (red arrows).

(4) HHQ is the immediate precursor of PQS [23], and both compounds play an important role in bacterial cell-to-cell communication [23] (yellow and blue arrows).

(5) PQS induces the expression of *pqsABCDE* [63], and is required for *phzA1-G1* expression, the gene responsible for PCN production (blue arrows). The release of PCN can induce neutrophil apoptosis and damage epithelial cells [64] (green arrows) allowing for immuno-evasion and deeper penetration of bacteria.

Dynorphin is a highly potent opioid peptide that is present in the enteric nervous system and has been shown to play a role in the regulation of intestinal motility, secretion, immune modulation, and maintenance of epithelial barrier function. The abundance of the neural network within the intestinal tract is matched only by its microbial flora, where bacterial cells outnumber the total number of cells in the body [14]. In this study, we found *κ*-opioid receptor agonists to induce PCN production in *P. aeruginosa*. Although dynorphin has been shown to be present in a variety of tissues, whether dynorphin accumulates in intestinal tissues following host stress and/or bacterial infection has not been previously addressed. Data from the present study show for the first time to our knowledge, that dynorphin is released into the intestinal lumen following I/R injury and accumulates in regions of high dynorphin concentration, such as on desquamated epithelium, where it binds to *P. aeruginosa*. Binding of dynorphin to *P. aeruginosa* was further confirmed in vitro by direct antibody staining. In addition, the penetration of dynorphin into the cytosolic compartment of bacterial cells was demonstrated by immunoelectron microscopy. These findings, coupled with the observation that dynorphin can activate the virulence of *P. aeruginosa*, may be of significant clinical relevance given that intestinal ischemia invariably accompanies physiologic stress and has been associated with fatal infection due to intestinal *P. aeruginosa* [43,44]. However, dynorphin may enhance the virulence of *P. aeruginosa* not only within the intestine but also at other sites of tissue injury and inflammation. There is overwhelming evidence that opioids are released and accumulate at sites of inflammation primarily because all of these tissues sites are heavily innervated and highly populated by macrophages and neutrophils. [45,46]. The ubiquitous presence of opioid receptors on nerves throughout the body suggests that exposure of *P. aeruginosa* to opioids may be one of the reasons it has evolved a mechanism to respond to these compounds [45,46]. Therefore, *P. aeruginosa* virulence could be activated by dynorphin in all infections associated with inflammation, including burns, implanted medical devices, and lung infections in patients with underlying lung disease. The precise mechanisms by which colonizing pathogens important to human disease process host signals for the purposes of virulence activation is a small and poorly understood area of investigation. The pathways by which bacteria gather, process, and become activated by host signals have been shown to be both specific to bacteria and specific to the host signal involved, and gene activation through the QS system has been previously reported [3,8]. A major mechanism by which the *κ*-opioid receptor agonists U-50,488 and dynorphin affect virulence gene activation in *P. aeruginosa* could be via expression of the *pqsABCDE* operon. The *pqsABCDE* operon directs the biosynthesis of 4-hydroxy-
2-alkylquinolines (HAQs), among which HHQ and PQS are themselves signaling molecules. Additionally, HHQ is a direct precursor of PQS [23]. PQS functions as a regulatory link between the LasR and RhlR quorum sensing systems [30,47], and has been shown to play a critical role in the pathogenesis of P. aeruginosa in nematodes, plants, and mice [24,48,49]. Data from the present study suggest that dynorphin synergizes with PQS to increase pqsABCDE expression. Further experiments are underway to clarify the precise mechanism of dynorphin activation of pqsABCDE expression.

Among critically ill and immunocompromised patients, infection with P. aeruginosa carries the highest case fatality rate of all nosocomial pathogens, approaching 60% [50,51]. The primary site of colonization for P. aeruginosa in such patients is the gastrointestinal tract, where as many as 50% of hospitalized patients harbor this organism [52,53]. Risk factors for mortality due to P. aeruginosa infection suggest that the degree of host stress is a major determinant of a fatal outcome from this pathogen [53,54]. In the present work we show (Figure 8) that P. aeruginosa presents a “triple threat” to its host when exposed to dynorphin in that it can 1) activate QS circuits via enhanced PQS production, 2) reduce populations of protective probiotic bacteria such as lactobacilli through the production of HQNO and PCN [55], and 3) increase the production of QS-dependent virulence gene products that affect host cell function such as PCN and the PA-I lectin/adhesin [30,56–58]. A clearer understanding of the mechanisms by which P. aeruginosa is activated to express virulence in direct response to host stress has the potential to lead to preventive therapies that interdict in the process of infection at its most proximate point.

Materials and Methods

Bacterial strains and culture conditions. P. aeruginosa strain PAO1 and its derivatives were routinely grown in tryptic soy broth (TSB) supplemented when necessary with tetracycline (Tc), 60 μg/ml gentamicin (Gm), 100 μg/ml, or carbenicillin (Cb), 300 μg/ml. P. aeruginosa strains PAO1 wild-type, ΔMvrR, ΔRhlR (Δvh), ΔgacA (ΔgacA), and ΔmvrR ΔgacA (Δvg) were kindly provided by B. Iglewski. Strains PAO6281 (ΔpqsA ΔpqsB ΔpqsC ΔpqsD ΔpqsE), ΔmvfR (ΔmvfR ISlacZ/hah, ID 13375) were obtained from the P. aeruginosa mutant library [59]. Strains PAO-R1 (ΔLasR(lasR::Tc)), PAO-JP-1 (ΔLasR(lasR::Tc)), and PDO100 (ΔRhlR (vh::Tnl501)) were kindly provided by B. Iglewski. Strains PAO6281 (ΔgacA (ΔgacA:Spir Sm)), PAO-MW1 (ΔRhlΔLasR (vh::Tnl501 lasEt)), and MP603 (ppcC::Tc) were kindly provided by C. Reimann, P. Greenberg, and C. Manoil, respectively.

PCN assay. P. aeruginosa cultures were grown at 37 °C, under shaking conditions at 220 rpm, in TSB supplemented with either morphone (Abbott Laboratories, http://www.abbott.com), U-50,488 (Sigma, http://www.sigmaaldrich.com), or BW373U86 (Sigma) at 50, 488, and 1-ml aliquots were serially collected. Cell density was measured at OD 600 nm, and samples were processed for PCN assay. In experiments in which strains complemented with ppG or gacA on the pUCP24 plasmid were used, experiments were performed with a higher dose of U-50,488 (1 mM), as a baseline of PCN production was already high due to these strains containing multi-copy plasmids encoding MvrR or GacA.

The β-galactosidase assay. Plasmid pGX5 [28] harboring the pqsA-lacZ fusion was introduced in P. aeruginosa strains PAO1 and MP603; and plasmids pGX1 harboring the mphR-lacZ fusion [23] and pMW303 harboring the mphABC-lacZ fusion [33] were introduced in PAO1 by electroperoration. Single colonies of PAO1(pGX5) or PAO1/pGX1, or PAO1/pMW303 were used to inoculate TSB supplemented with carbenicillin (Cb), 300 μg/ml, for overnight growth at 37 °C, 220 rpm. Overnight culture was diluted 1:100 in TSB, and 300 μg/ml Cb was supplemented with U-50,488, 200 μM, or dynorphin, 100 μM, or PQS, 100 μM, or without PQS. Aliquots of culture were taken out dynamically; pellet was collected by centrifugation at 6,000g, 5 min, and kept at −80°C before processing. MP603(pGX5) was inoculated in TSB supplemented with Tc, 60 μg/ml and Cb, 300 μg/ml. Overnight culture of these cultures was subgrown for 1 h to a cell density of OD600nm 0.07–0.1, and aliquoted to create the following groups (in triplicates): 1) “control”, 2) “dynorphin”, dynorphin added to a final concentration of 100 μM; 3) “PQS”, PQS added to final concentrations of 20 and 80 μM; and 4) “dynorphin + PQS”, dynorphin added to a final concentration of 100 μM, as described for 1 h, and then by the addition of the final concentration of 20 and 80 μM. All cell cultures were collected at the same time (5 h), cell density of 1:10 dilutes samples was measured at OD 600 nm, and cells were collected by centrifugation at 6,000g, 5 min, and kept at −80°C before processing. For β-galactosidase activity analysis, cells were treated with protein extractive reagent (Bug-Buster Master Mix; Novagen, http://www.emdbiosciences.com/htmlt/NVGHome.html) for 20 min at room temperature, centrifuged for 30 min at 4 °C, and cytosol fractions were used to measure β-galactosidase activity. In this case, 15 μl of cytosol fractions were loaded on 96-well plates, followed by 135 μl of a master mixture of o-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) containing 100 μl of 0.1 M Na-phosphate buffer (pH 7.5), 33 μl of ONPG, 4 mg/ml dissolved in 0.1 M Na-phosphate buffer (pH 7.5), and 2 ml of Mg buffer (63 μl of H2O, 35 μl of 2-mercaptoethanol, and 2 ml of 5 M MgCl2). Absorbance at 410 nm was measured using Plate Reader, and results were expressed as Miller units (1,000 × Δ410 nm × mg protein−1 × min−1).

RNA isolation and cDNA synthesis. For RNA isolation, 1 ml of P. aeruginosa PAO1 culture was grown in TSB with or without 200 μM U-50,488, to OD600nm = 3.0. Next, 2 ml of RNA Protect Bacteria reagent (Qiagen, http://www1.qiagen.com) was added immediately at the end of the incubation period, and samples treated as recommended by the Qiagen’s lysis protocol, followed by the addition of 3 ml of TRIzol LS reagent (Invitrogen, http://www.invitrogen.com). The RNA enrichment fraction was separated using Phase lock gel, heavy (Eppendorf, http://www.eppendorf.com). RNA was precipitated with isopropanol, dissolved in water, and the remaining DNA degraded using DNA-Free kit (Ambion, http://www.ambion.com). RNA integrity was monitored by formaldehyde agarose gel electrophoresis, and absence of DNA checked by PCR using primers for 16 s rRNA, forward 5'-GGACGGGTAGTAACTGCTA-3’ and reverse 5'-CTGAAAGGGCATGATGACCT-3’. The first-strand cDNA was prepared using 2 μg of total RNA, Superscript II RNase H−RT (Invitrogen), and random primers as recommended by the manufacturer’s protocol.

Real-time reverse transcription (RT–PCR) analysis. RT–PCR analysis was performed on the ABI Prism 7300 Sequence Detection System using SYBR Green qPCR SuperMix-UDG (Invitrogen), cDNA, and random primers: for glmA encoding citrate synthase (PA1580), 5’ C C A C T C T G C C A A G C A G A C A G A 3’ and 5’ T T T T G G C C G G T A G T T C G A G T C G G A 3’; for lasR encoding IL-1 (PA 2 5 7 0), 5’ C G A T G T C A T T C A C C A T G T C G 3’ and 5’ ACCCTGGACTATTGGGTG3’. The integrity of the RT–PCR products was confirmed by melting-curve analysis. Expression levels were calculated based on differences in Ct levels.

Protein concentration assay. Protein was measured using the BCA Protein Assay Reagent (Pierce, http://www.piercenet.com).

HHQ, HHQ, and PQS quantification. A single colony of PAO1 was used to inoculate 5 ml of TSB. Overnight culture was diluted in fresh TSB at 1:100, supplemented with either U-50,488 (200 μM) or dynorphin (100 μM). After 20 min of incubation under the same shaking conditions, cell cultures were aliquoted as 650 μl in 14-ml culture tubes. At designed time points, three tubes from each group were removed, and 650 μl of MeOH containing 2% acetic acid was immediately added; properly mixed, replaced into Eppendorf tubes, centrifuged at 15,000 g, and then processed to quantify HHQ, HHQ, and PQS by HPLCMS according to Lépine et al. [61]. Unlabeled PQS was obtained by the same synthetic route described for deuterium-labeled PQS [61]. The final PQS-d4 concentration was 20 mg/ml, and the stock solutions were in methanol.

C4-HSL and 3-oxo-C12-HSL quantification. Excreted C4-HSL and 3-oxo-C12-HSL were quantitated by LC/MS/MS using a water/acetonitrile gradient containing 1% acetic acid. The analyses were performed in positive electrospray ionization mode and the acquisitions were
obtained in Multiple Reaction Mode (MRM). The transitions monitored were m/z 298 to 102 for the N-(3-oxododecyl)-homoserine lactone and m/z 172 to 102 for the N-(butyryl)-homoserine lactone. Argon at 2.0 × 10−7 mtorr was the collision gas and the collision energy was 15 eV.

**Complementation of MvfR mutant with mvfR gene.** The mvfR gene was amplified using primers forward 5′-AAGGAGATAAGGGTGCC-TATTC-3′ and reverse 5′-CTACTCTGGTGCGGCGCTGG-3′ and cloned in pcR2.1 (Invitrogen). Plasmid pcR2.1/mvfR was digested with XbaI-HindIII restrictases, and mvfR was subcloned in pUC24 under the Phac promoter [52] to create pUC24/mvfR. The plasmids pUC24 and pUC24/mvfR were electroporated in strain 13375 defective in MvfR production to create strains MvR/pUC24 (control) and MvR/mvfR.

**Generation of GacA mutant with gacA gene.** The gacA gene was amplified using primers forward 5′-CTAGTGGCCGCAATGCTGCATCGC-3′ and cloned in pcR2.1 (Invitrogen). Plasmid pcR2.1/gacA was digested with XbaI-HindIII restrictases, and gacA was subcloned in pUC24 under the Phac promoter to create pUC24/gacA. The plasmids pUC24 and pUC24/gacA were electroporated in strain PAO2681 defective in GacA production to create strains GacA/pUC24 and GacA/gacA.

**Anti-MvfR antibody.** Polyclonal antisera against 50–63 peptide LVRGDKGYKEEQ of PAO1MVfR (Invitrogen, http://www.invitrogen.com) was produced in rabbits (ZYMED Laboratories, http://www.invitrogen.com). Anti-MvfR antibodies were affinity purified by AminoLink Plus Immobilization Kit (Pierce) using 50–63 peptide to create an affinity column.

**Segmental intestinal I/R model.** All experiments on mice were performed in accordance with University of Chicago guidelines and regulations, and mouse protocol number 71629 was approved by the Animal Care and Use Committee. Mice were fasted overnight prior to use in the I/R studies. For these studies, mice were lightly anesthetized with sevoflurane prior to an i.p. bolus injection of Avertin (Sigma-Aldrich). After 20–30 s, the mice were weighed and placed in a heating pad. A17.2 % solution, at 0.2 ml/kg body weight, mice were placed on a warmed heating pad under a warming light. An abdominal midline incision was made and the small intestines exposed. Sutures were placed so as to isolate a 20-cm segment of small intestine. The intestine was divided proximally and distally using cautery. A clamp was placed on the superior mesenteric artery (SMA) to occlude blood flow to the jejunum and ileum. Ischemia was induced by an overdose of anesthesia, followed by cervical dislocation. In this manner occurring in the presence of an opportunistic infection (I/R CFU/ml in 2 ml of PBS to recapitulate the clinical circumstance of I/R injury. Two negative controls were used: 1) grids without incubation with rabbit anti-dynorphin A 1–17 antibody, and 2) PAO1 grown in the absence of dynorphin.

**Biotinylation of dynorphin.** Dynorphin A (1–17) (Sigma) was biotinylated using NHS-PEO-biotin (Pierce), and purified by HPLC.

**Competitive ELISA for detection dynorphin in luminal flushings.** Luminal flushings were filtered with 0.22-μm filters (Millipore, http://www.millipore.com), aliquoted, and stored at −80 °C. Affinity purified P(ab)_2 of Frag Donk anti-Rb IgG (Jackson Immunological Research Laboratories, http://www.jax.org) at a concentration of 10 μg/ml in carbonate-bicarbonate buffer (Sigma) were coated onto Maxisorp immunomodules (Nunc, http://www.nuncbrand.com) for 2 h at 37 °C. Unbound sites were blocked with 0.1 % bovine serum albumin in PBS for 30 min at room temperature. After blocking, rabbit anti-dynorphin A 1–17 antibody (EMD Biosciences) at 1:25 dilution in 1 % BSA at humidified chamber for 3.5 h. Grids were extensively washed with PBS, blocked with 0.1 % BSA in PBS for 25 min in a humidified chamber, washed with PBS, and then covered with a 1:10 dilution of rabbit IgG conjugated with 10-nm gold particles (Ted Pella, http://www.tedpella.com) at 1:10 dilution in 0.1 % BSA. Grids were washed with PBS, fixed with 1 % glutaraldehyde in PBS for 10 min, washed with water, and stained briefly with uranyl acetate and lead citrate. Air dried grids were examined at 300 kV with an FEI Tecnai F30 (FEI, http://www.fei.com). Two negative controls were used: 1) grids without incubation with rabbit anti-dynorphin A 1–17 antibody, and 2) PAO1 grown in the absence of dynorphin.
200 μM and 100 μM, respectively. After 23 h of growth at 37 °C, 180 rpm, cultures were collected, centrifuged (5,000 rpm, 5 min) to remove cells, and supernatant was filtered using Millex-CV low protein binding membrane filters of 0.22-μm pore size (Millipore). The filtered supernatant, now termed conditioned media, was stored on ice before inoculation. L. plantarum and L. rhamnosus GG grown overnight in LRS (Oxoid, http://www.oxoid.com) at 37 °C were used to inoculate fresh MRS (2:100, vol/vol). Freshly inoculated culture, 10 μl was put into 96-well plates (Nunc), followed by an addition of 100 μl of TSB; TSB-U-50,488, 200 μl; TSB-dynorphin, 100 μl were added to wells. Plates with Lactobacillus spp. were incubated at 37 °C, unshaken, and growth was monitored dynamically by measuring OD 600 nm using Plate Reader.

Virulence assay of P. aeruginosa PAO1 using C. elegans. Wild-type N2 C. elegans were kindly provided by M. Glotzer, University of Chicago. Culturing, cleaning, egg preparation for synchronization, and transferring were performed according to “Maintenance of C. elegans” (http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html). For the experiment, two adult nematodes were transferred to a lawn of P. aeruginosa on NGM agar prepared by the following: P. aeruginosa PAO1 and PAO1 derivative AMVR mutant were grown for 6 h at 37 °C in TSB (control culture) or TSB supplemented with U-50,488 (U-50,488 culture); 200 μl, or TSB supplemented with dynorphin, 100 μM (dynorphin culture). Then, 10 μl of TSB media (control), or TSB supplemented with U-50,488, 1 mM, or dynorphin, 200 μM, was dropped at the center of NGM agar plate, allowed to dry for 10 min, and then 10 μl of bacterial culture was dropped on a respective plate (control culture on TSB spot, U-50,488 culture on U-50,488 spot, and dynorphin culture on dynorphin spot). The plates were incubated at 37 °C overnight and allowed to grow for 4 days to 20 °C, and 3 × 10 μl of TSB or TSB/U-50,488, 200 μM, were dropped periipherally at the lawn ring, daily. Total numbers of nematodes were counted daily.

Data analysis. Statistical analysis of the data was performed using Student’s t-test. Regression analysis was performed using Sigma plot software.

References
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