A. SPECIFIC AIMS

The opportunistic Gram-negative bacterium *Pseudomonas aeruginosa* (*P.a.*) is associated with multiple diseases including Cystic Fibrosis (CF), hospital-acquired pneumonia, and colonization of medical devices, among others[1]. *P.a.* is extremely aggressive and difficult to eradicate, especially when present in biofilms. A large number of *P.a.* factors required for virulence and biofilm formation are produced in response to environmental cues, and their expression is regulated using "quorum sensing" signals[2-6]. One of these signals is the *Pseudomonas* Quinolone Signal (PQS)[7]. In addition to acting as an inducer of virulence determinants, PQS exerts immune-modulating activities on the host, further facilitating infection by *P.a.*[8,9].

Regulation of PQS production occurs through the transcriptional activator PqsR. PqsR controls not only the biosynthesis of PQS, but is also required for full *P.a.* virulence, as deletion of *pqsR* leads to virulence attenuation in multiple hosts[10-13]. PqsR is a LysR-type transcriptional regulator (LTTR), and as such requires the binding of a small molecule, co-inducer, for full activity[14]. PQS, and its immediate precursor HHQ (2-heptyl-4-quinolone), act as co-inducers of PqsR, mediating virulence[12]. Conformational switching in response to a co-inducer molecule constitutes the basis for transcriptional activation by LTTR proteins. However, there is no structural information describing these transitions in PqsR.

Importantly, PqsR can also intercept signals released by the host during stress (the κ-opioid dynorphin A), and use them for augmentation of *P.a* virulence[15]. The mechanism of this activation is currently unknown. Here we propose to: (i) structurally characterize PqsR and the conformational changes that it undergoes upon co-inducer binding, in order to obtain an atomic-level description of PqsR-mediated transcriptional activation; and (ii) study the basis for PqsR activation by the host κ-opioid dynorphin A. These are key steps toward understanding the molecular mechanism of activation of the global virulence regulator PqsR by both bacterial and host signals. PqsR regulates a vast array of virulence-related genes, and thus is a logical target for drug design. The synthesis of PQS antagonists that compete for binding to PqsR, and lock the protein in its inactive conformation, will benefit immensely from the structural information on PqsR obtained here.

Specific Aim 1: Determine the mechanism of PqsR conformational switching in response to its co-inducers. The main goal in this part of the proposal is to characterize the conformational changes that PqsR experiences upon co-inducer binding. We will target structure determination of unliganded and PQS (or HHQ)-bound protein in parallel. The results will lead to understanding of the molecular mechanism of PqsR-mediated transcriptional activation, and will provide a solid basis for drug development. In addition, results obtained in this specific aim will greatly advance our knowledge about one of the biggest families of transcriptional regulators in bacteria, the LTTR family. The use of CoFi methodology[16, 17] will allow for the generation of multiple protein constructs for crystallization screening. Extensive crystallization screening of several proteins/protein-ligand complexes simultaneously is possible using available instrumentation. The approach proposed here, together with our expertise in X-ray crystallography[18-21], will ensure successful completion of the proposed research.

Specific Aim 2: Characterize the mechanism of host-induced PqsR activation. Here we will focus on characterizing PqsR activation induced by the host κ-opioid peptide dynorphin A (dynA). DynA synergizes with PQS via PqsR leading to increased production of alkyl-quinolones (AQs), pyocyanin, and to enhanced virulence[15]. However, the mechanism of this activation is currently unknown. We will use a battery of assays to test the hypothesis that dynA binds directly to PqsR. Two possibilities will be tested: (i) dynA binds to the PqsR-PQS complex stabilizing the "active" protein conformation, and (ii) dynA increases the affinity of the protein for its co-inducer, PQS, leading to amplification of transcriptional activation. Structural characterization of the peptide-protein complex is proposed as a follow-up to the binding studies. Results obtained in this specific aim will provide a breakthrough in our knowledge of how *P.a.* intercepts host-released molecules at sites of inflammation to switch to a more virulent phenotype.

The work proposed here will break new ground in our understanding of the molecular mechanisms of virulence regulation by both bacterial and host signals. It will provide a solid platform for the design of inhibitors of PqsR and PQS-related virulent phenotypes.