Recognition of Host Immune Activation by Pseudomonas aeruginosa
Author(s): Licheng Wu, Oscar Estrada, Olga Zaborina, Manjeet Bains, Le Shen, Jonathan E. Kohler, Nachiket Patel, Mark W. Musch, Eugene B. Chang, Yang-Xin Fu, Michael A. Jacobs, Michael I. Nishimura, Robert E. W. Hancock, Jerrold R. Turner and John C. Alverdy
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Recognition of Host Immune Activation by *Pseudomonas aeruginosa*

Licheng Wu,[1] Oscar Estrada,[1] Olga Zaborina,[1] Manjeet Bains,[4] Le Shen,[2] Jonathan E. Kohler,[1] Nachiket Patel,[1] Mark W. Musch,[3] Eugene B. Chang,[3] Yang-Xin Fu,[2] Michael A. Jacobs,[5] Michael I. Nishimura,[1] Robert E. W. Hancock,[4] Jerrold R. Turner,[2] John C. Alverdy[1]*

It is generally reasoned that lethal infections caused by opportunistic pathogens develop permissively by invading a host that is both physiologically stressed and immunologically compromised. However, an alternative hypothesis might be that opportunistic pathogens actively sense alterations in host immune function and respond by enhancing their virulence phenotype. We demonstrate that interferon-γ binds to an outer membrane protein in *Pseudomonas aeruginosa*, OprF, resulting in the expression of a quorum-sensing dependent virulence determinant, the PA-I lectin. These observations provide details of the mechanisms by which prokaryotic organisms are directly signaled by immune activation in their eukaryotic host.

Although opportunistic infection has been traditionally viewed as a passive phenomenon in which exploitative pathogens invade a weakened host, recent advances in the understanding of bacterial virulence gene regulation would suggest that this process is much more complex than previously appreciated (*1*). Because bacteria are constantly assessing the cost versus benefit of expressing virulence (*2*), it is logical that they might develop a contingency-based system to recognize physiological and immunological disturbances in their hosts. Although host cells are known to express receptors that bind bacteria for the purpose of activating the immune system, it must be considered that bacteria themselves might possess specialized receptors that in turn recognize and respond to host immune activation.

Departments of [1]Surgery, [2]Pathology, [3]Medicine, University of Chicago, Pritzker School of Medicine, Chicago, IL 60637, USA. [4]Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC V6T1Z4, Canada. [5]Department of Genome Sciences and Medicine, University of Washington, Seattle, WA 98195, USA.

*To whom correspondence should be addressed. E-mail: jalverdy@surgery.bsd.uchicago.edu

We studied this possibility using the human opportunistic pathogen, *Pseudomonas aeruginosa*, because its virulence gene regulation is well studied. We used the type I *P. aeruginosa* lectin (PA-I or *lecA*), an adhesin of *P. aeruginosa*, as a representative readout for virulence expression in this organism. Previously, we demonstrated that within the intestinal tract of a stressed host, the lethality of *P. aeruginosa* is dependent on the expression of the PA-I lectin, which causes increased permeability to its lethal cytotoxins across the intestinal epithelium (*3*). PA-I has also been shown to induce apoptosis in respiratory epithelial cells, which suggests that PA-I may be directly cytotoxic to epithelial cells (*4*). Finally, the expression of PA-I (*lecA*) is dependent on the quorum-sensing (QS) signaling system (*5*), a core system of virulence gene regulation that controls multiple virulence genes in *P. aeruginosa*.

We considered that immune elements might directly activate the virulence of *P. aeruginosa*. As a physiologically relevant in vitro source of such immune factors, supernatants from antigen-stimulated T cells, which express an array of cytokines (*6*), were evaluated for their ability to increase PA-I expression in *P. aeruginosa* strain PLL-EGFP/27853, a PA-I-GFP reporter (*7*) that was readily available and verified in a previous report by our laboratory (*8*). PA-I expression was increased by supernatant from activated T cell cultures, as assessed by enhancement of fluorescence in the PA-I-GFP fusion reporter strain (Fig. 1A), but not in controls. To determine whether this effect was due to specific cytokines, the reporter strain was individually exposed to human IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α). Of these, only IFN-γ induced a significant increase in PA-I expression that started at early stationary phase of growth (Fig. 1B). None of the cytokines tested had any significant effect on bacteria growth (Fig. 1C). Immunodepletion of IFN-γ resulted in the complete loss of its PA-I inducing capacity (Fig. 1A), which suggests that IFN-γ was the critical component in the activated T cell media that induced PA-I expression (Fig. 1A). Next, we examined PA-I expression in the completely genomically sequenced strain of *P. aeruginosa*, PAO1 (*9*), after exposure to human recombinant IFN-γ, TNF-α, IL-2, IL-4, IL-8, and IL-10 (*7*). Northern blot analysis revealed that only IFN-γ was capable of inducing *lecA* gene transcription (Fig. 1D).

Virulence in *P. aeruginosa* is highly regulated by the QS signaling system, a hierarchical system of virulence gene regulation that is dependent on bacterial cell density and growth phase (*10–12*). To determine the effect of growth phase on the response of *P. aeruginosa*

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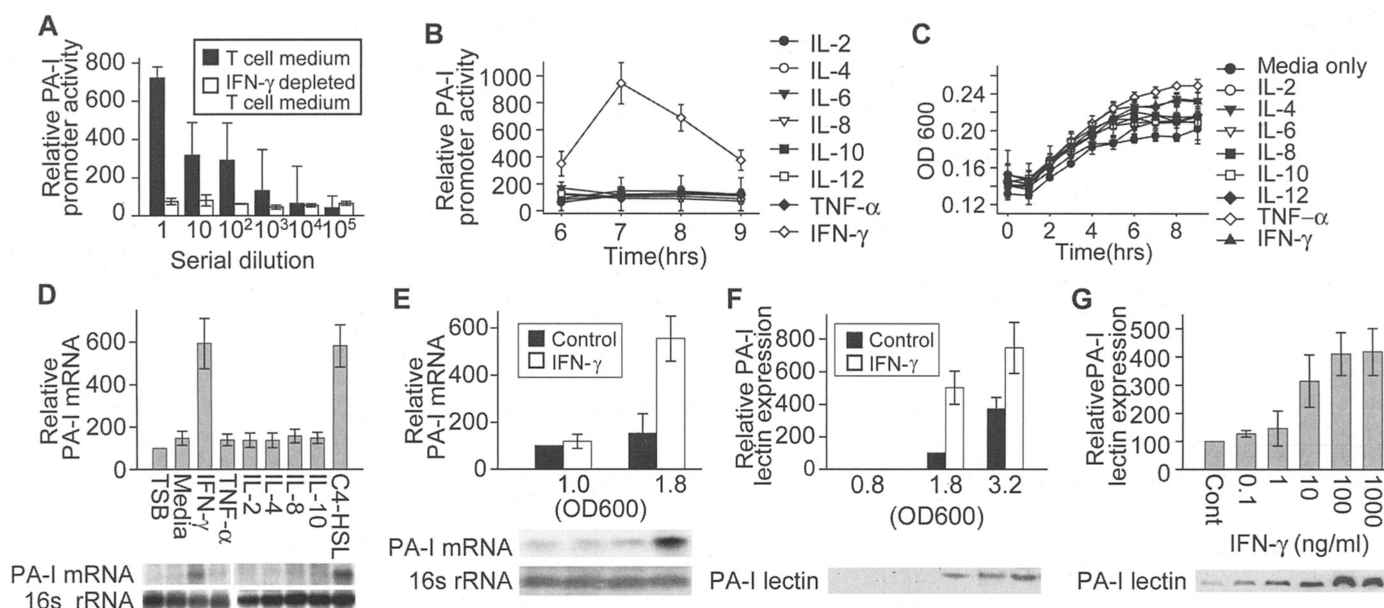

Fig. 1. IFN-γ induces the expression of the PA-I lectin in *P. aeruginosa*. Error bars, mean ± SD. **(A)** PLL-EGFP/27853 was exposed to media from activated T cells, and PA-I expression was assessed. Immunodepletion of the media with antibody to IFN-γ abolished the PA-I inducing effect by activated T cell culture media (maximum at 7 hours). **(B)** Only IFN-γ induced PA-I promoter activity after exposure of various cytokines to the GFP-PA-I reporter strain. **(C)** Optical density of *P. aeruginosa* showed that *P. aeruginosa* reached stationary growth phase at 6 hours. **(D)** *P. aeruginosa* (PAO1) was incubated with 200 ng/ml IFN-γ, TNF-α, IL-2, IL-4, IL-8, and IL-10 in cell culture media for 4 hours, and PA-I mRNA was measured by Northern blot. Induction of PA-I mRNA was observed only in the presence of IFN-γ and C_4-HSL. **(E)** *P. aeruginosa* harvested at 2 hours (OD_{600} = 1.0) and 4 hours (OD_{600} = 1.8) in the presence of 200 ng/ml IFN-γ in cell culture media. Northern blotting demonstrated that PA-I mRNA was significantly increased at early stationary phase of growth (OD_{600} = 1.8). **(F)** PA-I expression was induced following exposure to IFN-γ during stationary phase of growth, an effect not observed during log phase of growth. **(G)** Dose-dependent enhancement of PA-I expression after exposure to IFN-γ for 6 hours.

to IFN-γ, bacteria were harvested at various growth phases after exposure to IFN-γ, and PA-I mRNA and protein were measured (*7*). Both transcription and translation of PA-I increased in response to IFN-γ, starting at early stationary phase of growth (Fig. 1E and F). PA-I protein expression was also dose dependent (Fig. 1G). Taken together, these results suggest that PA-I expression in *P. aeruginosa* is enhanced in the presence of IFN-γ in a growth-dependent manner.

To determine whether IFN-γ induced PA-I by activation of the QS signaling system, we measured *rhlI* gene expression in response to IFN-γ (*7*). *RhlI* is the gene required for the synthesis of C_4-HSL (C_4-homoserine lactone), a core QS signaling molecule that plays a central role in the expression of PA-I (*5*). IFN-γ induced *rhlI* transcription in PAO1 (Fig. 2, A and B), and C_4-HSL synthesis increased significantly after exposure to IFN-γ (Fig. 2C). Activation of the QS system by IFN-γ also led to the increase of pyocyanin (PCN), another QS-dependent virulence product (*13*) (Fig. 2D). In addition, *rhlI* and *rhlR* were required for the production of PCN and PA-I expression in response to IFN-γ, because the increase of these two virulence factors by IFN-γ was abolished in mutant strains (Fig. 2, E and F). Finally, supernatant from *P. aeruginosa* exposed to IFN-γ, but not controls, altered the barrier function of cultured epithelial cells (fig. S1). Taken together, these data suggest that the QS system plays a key role in the response of *P. aeruginosa* to IFN-γ and that IFN-γ can shift the virulence of *P. aeruginosa* against epithelial cells.

We next hypothesized that IFN-γ may directly bind to a protein on the surface of *P. aeruginosa*, leading to virulence up-regulation. Consistent with this, we observed that IFN-γ avidly bound to whole fixed cells of *P. aeruginosa* in a dose-dependent manner (Fig. 3A). The vast majority of bacterial cells (73% ± 3.2% versus 8.5% ± 2.5%) bound IFN-γ (Fig. 3B and fig. S2). The binding capacity of the IFN-γ to *P. aeruginosa* was not affected significantly by the growth phase of bacteria (fig. S3A). To determine whether IFN-γ bound to membrane or cytosolic fractions of *P. aeruginosa*, equal protein concentrations of each fraction were prepared (*7*), and results showed that IFN-γ preferentially bound to membrane fractions by enzyme-linked immunosorbent assay (ELISA) (fig. S3B). Furthermore, IFN-γ binding to *P. aeruginosa* membranes was diminished upon proteinase K treatment (fig. S3C), which suggests that IFN-γ binds to a protein on the bacterial cell membrane. Binding was specific to IFN-γ, because no binding was observed with any other cytokines tested (fig. S3D). Taken together, these data indicate that IFN-γ binds specifically to a membrane protein (s) on *P. aeruginosa*.

P. aeruginosa membrane proteins solubilized with mild detergents (*7*) retained their binding capacity to IFN-γ (Fig. 3C), thus making it possible to isolate the putative binding protein by immunoprecipitation. Membrane proteins were next separated by nondenaturing gel electrophoresis, transferred to polyvinylidene difluoride membranes, and hybridized with IFN-γ followed by biotin-labeled antibody to IFN-γ; results revealed a single immunoreactive band at 35 kD that was dependent on the dose of IFN-γ (Fig. 3D). Immunoprecipitation against the *P. aeruginosa* fractionated membrane protein isolated a 35-kD protein that was IFN-γ dependent (Fig. 3E). Use of ESI-TRAP LC-MS-MS ion trap (electrospray ionization–telomeric repeat amplification protocol liquid chromatography tandem mass spectrometry) identified the 35-kD protein to be the *P. aeruginosa* outer membrane porin OprF (Fig. 3F) (*14*). We next verified that OprF was a major binding site for IFN-γ by showing that solubilized membrane proteins from OprF mutant strains (*15*) displayed reduced binding to IFN-γ (Fig. 4A). Immunoprecipitation of solubilized membrane protein confirmed the role of OprF by showing complete loss of the ~35-kD band in the OprF mutant strain (Fig. 4B). Further evidence supporting the role of OprF in the IFN-γ response was found when mutant strains failed to increase PA-I protein expression after exposure to an effective stimulating dose of IFN-γ as compared with the wild-type strain (Fig. 4, C and D). When OprF was reconstituted in the mutant strain 31899 using the plasmid pUCP24/OprF,

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responsiveness to IFN-γ was reestablished, with an increase in PA-I protein expression (Fig. 4E). Finally, ELISA binding assays between purified OprF and IFN-γ demonstrated that OprF binds directly to human IFN-γ (Fig. 4F) in a dose-dependent manner.

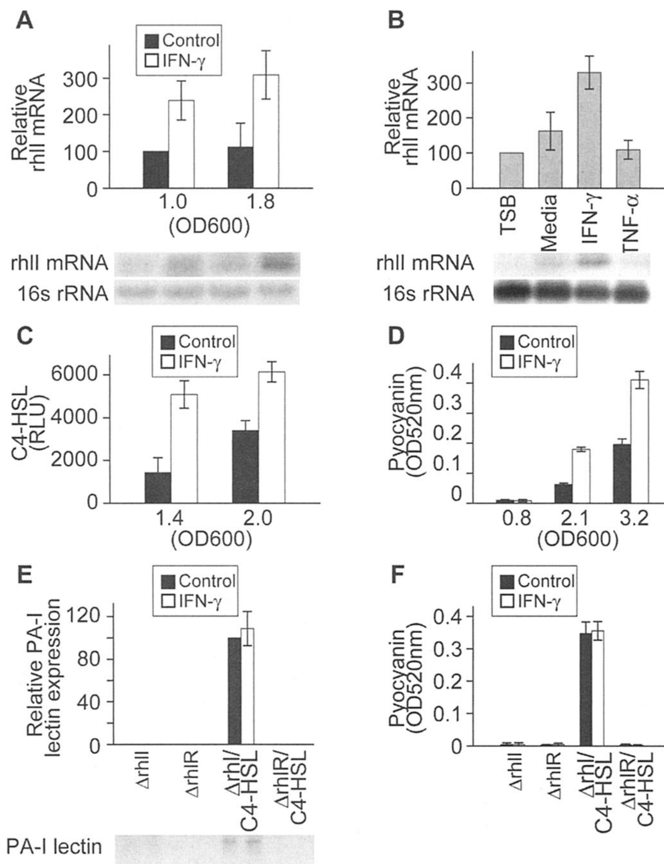

Fig. 2. The presence of *rhlI* and *rhlR*, core QS signaling elements in *P. aeruginosa*, are required for PA-I expression and pyocyanin production in response to IFN-γ. Error bars, mean ± SD. **(A)** *P. aeruginosa* harvested at 2 hours (OD_{600} = 1.0) and 4 hours (OD_{600} = 1.8) after incubation with 200 ng/ml IFN-γ in cell culture media. Northern blotting demonstrated that IFN-γ increased *rhlI* mRNA levels significantly. **(B)** IFN-γ, but not TNF-α, induced the transcription of *rhlI* mRNA. **(C)** The gene product of *rhlI*, C_4-HSL, a key diffusible QS signaling molecule, was measured by the luminescence reporter strain pSB536 in PAO1 supernatant and was increased following exposure to IFN-γ. **(D)** Pyocyanin, an additional QS-dependent virulence factor, was also up-regulated in PAO1 in the presence of 100 ng/ml of IFN-γ. **(E)** Immunoblots of PA-I expression demonstrated that exposure of *rhlI* and *rhlR* mutants to IFN-γ failed to induce PA-I expression. The addition of exogenous C_4-HSL did not restore responsiveness to IFN-γ in either mutant. **(F)** Pyocyanin production by IFN-γ required the presence of *rhlI* and *rhlR*, because mutants did not produce pyocyanin when exposed to IFN-γ. Adding C_4-HSL to the *rhlI* and *rhlR* mutants did not restore responsiveness to IFN-γ.

Although it was previously recognized that various cytokines, including IL-1β (*16*) and TNF-α (*17*), can affect the growth and virulence properties of bacteria, little progress had been made on the mechanistic details of these initial observations. For example, although TNF-α has been shown to display high-affinity binding to *Shigella flexneri*, the specific receptor that mediates this response has not yet been identified. In addition, although TNF-α has been shown to induce *Shigella flexneri* to become more invasive against cultured epithelial cells, the specific signaling pathways and virulence factors that mediate this response remain unknown. The observation here that *P. aeruginosa* binds INF-γ through OprF, resulting in activation of the QS system, provides specific information on the mechanisms by which certain bacteria sense and respond to the host immune system. That IFN-γ binding to OprF induces *P. aeruginosa* to express both PA-I and pyocyanin, both of which are capable of disrupting epithelial cell function, is intriguing given that, in contradistinction to TNF-α, the main function of IFN-γ is bacterial clearance (*18*). Data from the present study provide molecular evidence that certain opportunistic pathogens such as *P. aeruginosa* may have evolved a contingency-based mechanism to mount an effective countermeasure to immune activation by their host. It remains to be clarified whether other virulence regulators that activate the QS system, such as the quinolone signaling system (*19*, *20*) and the lasRI system (*5*), may be involved in the transduction of membrane signaling in *P. aeruginosa* to IFN-γ. Finally, the design of appropriate animal models using IFN-γ knockout mice and OprF mutant strains will be

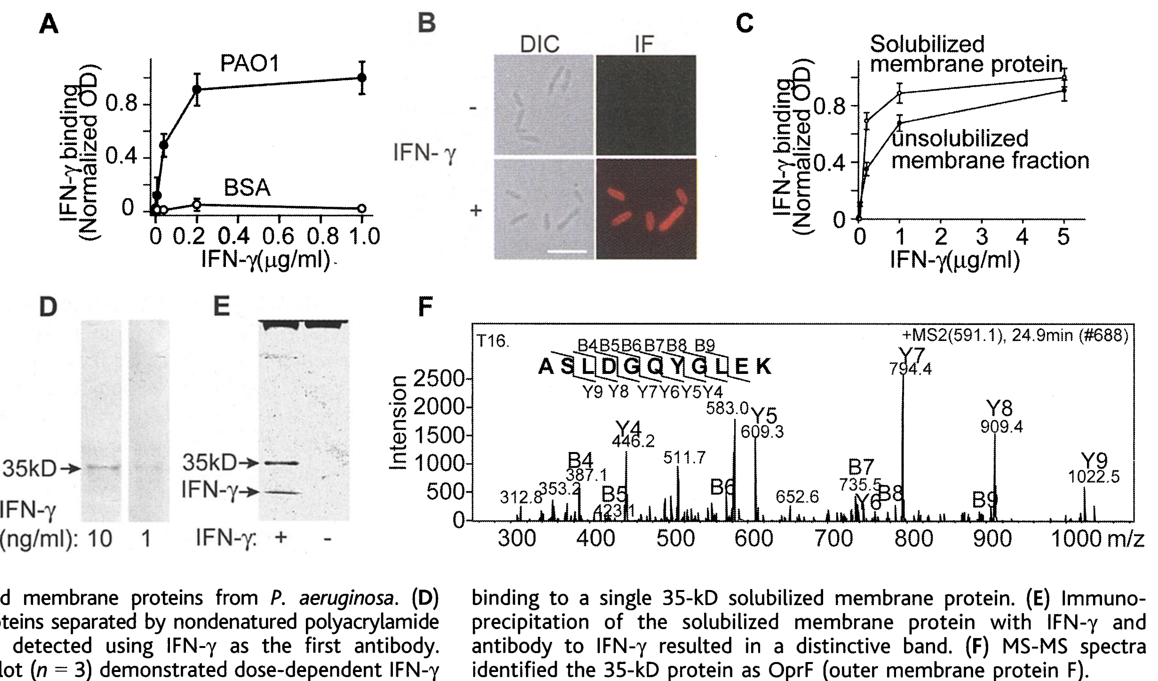

Fig. 3. Identification of the IFN-γ binding site from solubilized membrane fractions of *P. aeruginosa* (PAO1). Error bars, mean ± SD. **(A)** ELISA assay demonstrated a dose-dependent binding of IFN-γ to *P. aeruginosa*. **(B)** Epifluorescence photomicrographs of IFN-γ bound to whole cells of *P. aeruginosa*. Binding was detected using biotin-labeled antibody to IFN-γ and fluorescence Alexa 594–labeled streptavidin. DIC, digital intense phase contrast; IF, immunofluorescence; scale bar, 5 μm. **(C)** ELISA assay demonstrated that IFN-γ binds to solubilized membrane proteins from *P. aeruginosa*. **(D)** Solubilized membrane proteins separated by nondenatured polyacrylamide gel electrophoresis were detected using IFN-γ as the first antibody. Representative immunoblot (*n* = 3) demonstrated dose-dependent IFN-γ binding to a single 35-kD solubilized membrane protein. **(E)** Immunoprecipitation of the solubilized membrane protein with IFN-γ and antibody to IFN-γ resulted in a distinctive band. **(F)** MS-MS spectra identified the 35-kD protein as OprF (outer membrane protein F).

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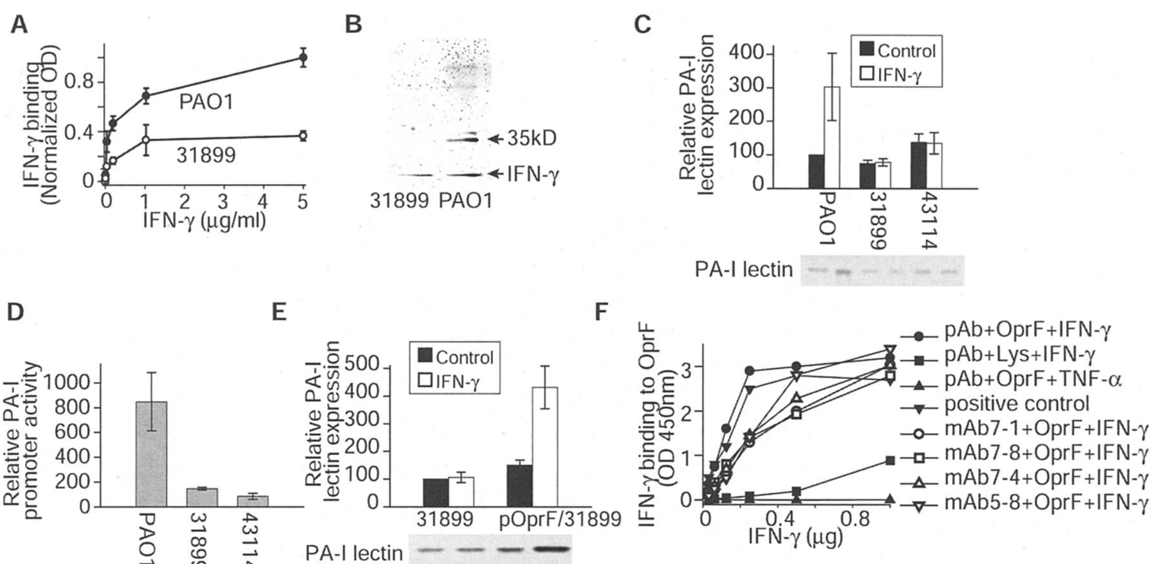

Fig. 4. IFN-γ binds to OprF and induces PA-I expression. Error bars, mean ± SD. **(A)** ELISA binding assays of IFN-γ to solubilized membrane protein from wild-type *P. aeruginosa* (PAO1) and the OprF knockout mutant strain 31899 showing attenuated IFN-γ binding to the solubilized membrane protein from the mutant strain. **(B)** Immunoprecipitation of solubilized membrane proteins from OprF mutant strain 31899 with IFN-γ, demonstrating absence of the 35-kD band seen with the parent wild-type strain (PAO1). **(C)** PA-I protein expression measured by immunoblot in wild-type (PAO1) and mutant strains (31899, 43114) exposed to 200 ng/ml IFN-γ, demonstrating an inability of IFN-γ to enhance the expression of PA-I in the OprF mutant strains. **(D)** Wild-type strain (PAO1) and OprF mutant strains (31899, 43114) carrying the GFP-PA-I fusion plasmid were incubated with 200 ng/ml IFN-γ, and fluorescence was assessed over time. Results demonstrate a lack of enhanced PA-I expression in mutants exposed to IFN-γ. **(E)** Reconstitution of OprF in mutant strain 31899 demonstrating reestablishment of the responsiveness of PA-I expression to IFN-γ. **(F)** Antibody to OprF (polyclonal, pAb; monoclonal, mAb) was coated onto microtiter plate. The complexes [OprF and IFN-γ, IFN-γ and Lys (lysozyme), and OprF and TNF-α] were added and detected by biotin-labeled antibody to IFN-γ. ELISA assay demonstrated that human IFN-γ binds to purified OprF. Results are a representative experiment of three independent studies.

necessary to confirm the role of IFN-γ binding to OprF on *P. aeruginosa* virulence in vivo.

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A Phenylalanine Clamp Catalyzes Protein Translocation Through the Anthrax Toxin Pore

Bryan A. Krantz,[1]* Roman A. Melnyk,[1]* Sen Zhang,[1] Stephen J. Juris,[1] D. Borden Lacy,[1] Zhengyan Wu,[2] Alan Finkelstein,[2] R. John Collier[1]†

The protective antigen component of anthrax toxin forms a homoheptameric pore in the endosomal membrane, creating a narrow passageway for the enzymatic components of the toxin to enter the cytosol. We found that, during conversion of the heptameric precursor to the pore, the seven phenylalanine-427 residues converged within the lumen, generating a radially symmetric heptad of solvent-exposed aromatic rings. This "ϕ-clamp" structure was required for protein translocation and comprised the major conductance-blocking site for hydrophobic drugs and model cations. We conclude that the ϕ clamp serves a chaperone-like function, interacting with hydrophobic sequences presented by the protein substrate as it unfolds during translocation.

Anthrax toxin is composed of three nontoxic proteins, which combine on eukaryotic cell surfaces to form toxic, noncovalent complexes. [See (*1*) for a review.] Protective antigen (PA), the protein translocase component, binds to a cellular receptor and is activated by a furin-family protease. The resulting 63-kD receptor-bound fragment, PA_{63}, self-assembles into the prepore, which is a ring-shaped homoheptamer (Fig. 1A). The prepore then forms complexes with the two ~90-kD enzymatic components, lethal factor (LF) and edema factor (EF). These complexes are endocytosed and delivered to an acidic compartment (*2*). There, the prepore undergoes an acidic pH-dependent conformational rearrangement (*3*) to form an ion-

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