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Q&A

How did you become involved in doing research?
I transferred to KU from a community college that did not emphasize research experience at all, so I was surprised and excited by the emphasis on research at KU. I contacted Dr. Lamb through email, expressing interest in the work she was doing, and was invited into her lab for a trial period. I fell in love with the research, and was excited to work on my own project.

How is the research process different from what you expected?
In order to scratch the surface of the research I was doing, I had to do a lot of research in the literature. I was definitely surprised by how much reading (and rereading!) it took for me to really get a better understanding of my research topic.

What is your favorite part of doing research?
Research is an inquiry driven field—I enjoy the process of asking a question and actively seeking an answer. I am so impressed by the ingenuity of the whole research process.

Isolation and crystallization of PvdJp2, a non-ribosomal peptide synthetase domain in *Pseudomonas aeruginosa*

Betsy Ramirez

ABSTRACT

During times of iron deprivation, many bacteria produce low molecular weight compounds called siderophores, which have the ability to scavenge iron from the host environment. Pyoverdin is a siderophore produced by *Pseudomonas aeruginosa* during extreme iron starvation. The biosynthesis of pyoverdin involves several multi-modular non-ribosomal Peptide Synthetases (NRPSs). The NRPS PvdJ incorporates two amino acids into the growing pyoverdin. PvdJp2 is a structural domain within the second module of PvdJ that acts as a scaffold and tether for the incorporation of formyl-hydroxyornithine. Present work is focusing on the expression, purification, and crystallization of PvdJp2. PvdJp2 was determined to express best after induction at 30°C for 22 hours, and is purified using standard chromatography techniques, exploiting the His-tag. Current methods of crystallization have not yet yielded diffraction quality crystals. Future work will focus on alternative methods of crystallization, such as increasing the protein concentration and changing the incubation temperature.

INTRODUCTION

Bacterial pathogens pose a major risk to the human population due to the increasing prevalence of multi-drug resistant bacterial strains. The opportunistic pathogen *Pseudomonas aeruginosa* is a Gram negative rod that attacks hosts with weakened immune systems. *P. aeruginosa* causes 10-15% of hospital-acquired infections worldwide due to the breakdown of physical barriers and high numbers of immunocompromised or elderly patients in hospitals (Strateva and Yordanov, 2009). Examples of typical *Pseudomonas* patients are burn victims, cystic fibrosis patients, and AIDS patients. *Pseudomonas* infections have been found in 80% of cystic fibrosis patients over 18 and are considered to be a major factor in their mortality (Conova, 2003). In addition, *P. aeruginosa* bacteremia carries a 31% mortality rate (Tacconelli et al., 2002)

As a significant hospital-acquired pathogen, *Pseudomonas* possesses many antibiotic resistance strategies—both innate and adapted from the environment (Strateva and Yordanov, 2009). *P. aeruginosa* intrinsically contains efflux pumps to pump out antibiotics almost as quickly as they are imported in. In addition, *P. aeruginosa* contains AmpC Beta-lactamase, which targets Penicillin-derived antibiotics by breaking apart a signature bond in their unique structure. *Pseudomonas* also has the ability to acquire resistance from the environment through horizontal gene transfer—which is the uptake of genetic material from the environment. It has been reported by Pechere and Kohler (1999) that *P. aeruginosa* is considered a "phenomenon" for having demonstrated almost all known enzymatic and mutational mechanisms for resistance. Multi-Drug resistance has emerged in 27-72% of patients infected with initially susceptible *Pseudomonas* isolates (Obritsch, Fish, MacLaren, and Jung, 2005).

One of the virulence factors that *Pseudomonas* possesses is the ability to produce siderophores, low molecular weight compounds released by the bacterium into the environment to scavenge iron for metabolic purposes. Iron, while abundant in the human body, is typically bound to hemoglobin, myoglobin, or cytochrome (Jurado, 1997). Additional iron is held by iron-sequestering proteins such as transferrin, lactoferrin, and ferritin. Therefore, the amount of free iron within the host is much less than what the bacteria requires to carry out its functions. In response to this, many bacteria have developed strategies to scavenge iron from the hosts' environment for essential metabolic processes (Jurado, 1997).

P. aeruginosa produces two types of siderophores: pyoverdin and pyochelin (Dumas, Ross-Gillespie, Kummerli, 2013). Pyoverdin production is energetically expensive, but highly efficient. Pyochelin is energetically inexpensive, but not as efficient. Pyoverdin is produced, therefore, during extreme iron deprivation, while pyochelin is produced during less extreme iron limitation. It has been found that alternating between these two siderophores allows the bacteria to survive in a wide range of conditions (Dumas et al., 2013). In this paper, the focus will be on pyoverdin biosynthesis.

Many siderophore biosynthesis pathways utilize non-ribosomal Peptide Synthetases (NRPSs), which are currently a hot topic in research. As indicated by their name, these enzymes do not use ribosomes to create proteins. Ribosomes are almost exclusively used to create proteins in nature, and work by binding to a specific template and translating the message from one biological "language" to another. This translation results in a protein product. Because they use a template, ribosomes must adhere to very specific rules regarding the types of molecules they will interact with. Alternatively, NRPSs are composed differently and therefore have more freedom to work with diverse molecules. Therefore, bioactive peptides generated by NRPS enzymes are incredibly diverse structurally due to their ability to incorporate unusual amino acids into their product. These amino acids can then be further modified by other biological processes (Mootz et al., 2002).

Non-ribosomal Peptide Synthetases are independent of nucleic acid templates and instead work by coordinating catalytic centers and using assembly-line production (Marahiel and Essen, 2009). Each module is responsible for a specific addition to the final peptide

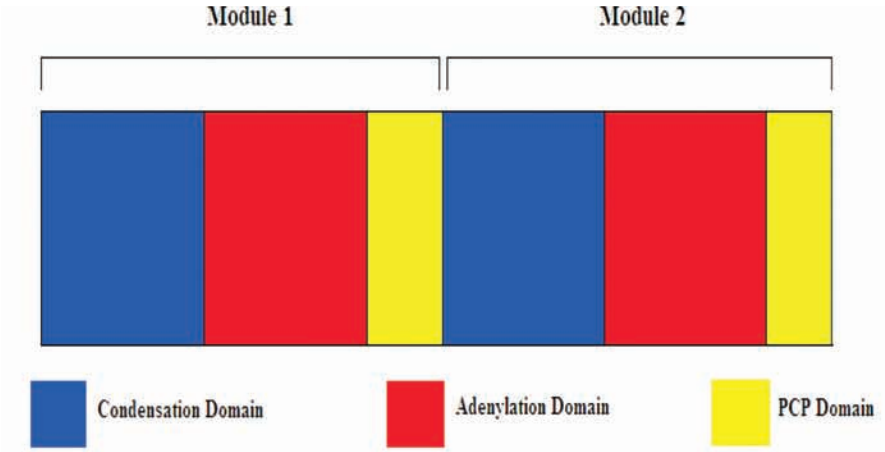


Fig. 1. Sequence of PvdJ non-ribosomal Peptide Synthetase domains. Module 1 incorporates Lysine, and Module 2 incorporates formyl-hydroxyornithine into the growing pyoverdine (Ravel and Cornelis 2003).

product. Each module contains three principle domains: the Adenylation (A) domain, which recognizes and activates the amino acid, the Peptidyl Carrier (PCP) domain, which acts as a scaffold and tether to the activated amino acid, and the Condensation (C) domain, which catalyzes peptide bond formation to join neighboring amino acids (Marahiel and Essen, 2009). The terminal module also may contain an additional domain, the Thioesterase domain, which releases the finished peptide from the enzyme (Ravel and Cornelis, 2003).

The protein domain studied in this paper is PvdJp2, a domain of a linear NRPS involved in pyoverdin biosynthesis in *Pseudomonas aeruginosa*. PvdJp2 is a PCP domain of an NRPS that catalyzes the addition of the traditional amino acid lysine and non-traditional amino acid formyl-hydroxyornithine into the growing pyoverdin chain. Linear NRPSs have the three aforementioned core domains arranged as C – A – PCP in an "elongation module" (Mootz et al., 2002) (Fig. 1). The peptides produced by linear NRPSs are entirely dependent on the order and the number of modules involved in the pathway, and therefore carry the possibility for creating novel products by the process of "module swapping" (Mootz et al., 2002).

The overall goal of this project was to characterize the structure of the protein domain PvdJp2. Understanding the structure of proteins allows for researchers to seek further information about how they interact with other molecules and substrates. In order to determine the structure, several steps must be taken. The protein must first be grown in very specific conditions that will allow optimum expression. These conditions vary depending on the protein, and therefore must be determined by methodical testing. Once over-expression patterns are determined for the protein, it must be purified from other proteins. The purified protein is then subjected to crystallization procedures, which can potentially induce the precipitation of the protein out of solution in a crystalline pattern. The protein crystal can then be collected and analyzed with X-Ray crystallography in order to create a 3-dimensional structure using computer analysis.

METHODS

See online appendix at http://ugresearch.ku.edu/Ramirez

RESULTS

The N-PvdJp2 protein overexpression plasmid was obtained from Dr. Kathleen Meneely. All further work on the overexpression and purification of N-PvdJp2 was completed by the author.

In order to determine the optimum conditions for N-PvdJp2 overexpression, an induction test was performed (Fig. 1). Results from the induction test indicated that two conditions led to optimal expression of N-PvdJp2:

1. Overnight growth at 15°C after being induced.
2. Overnight growth at 30°C after being induced.

Based on the relative darkness of the bands on the gel, it appeared as though growth at 30°C yielded slightly more protein at the appropriate molecular weight. All further tests with N-PvdJp2 were grown under these conditions.

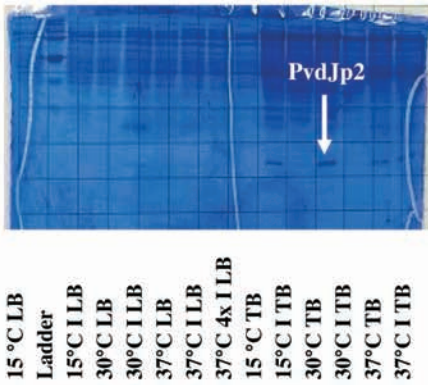
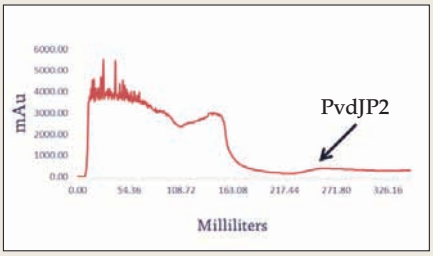


Fig. 2. Coomassie stained SDS-PAGE

The purification process was carried out using standard chromatography techniques, exploiting the His-tag. Chromatography is a collective term used to describe the different processes used to separate mixtures. The Nickel column binds the His-tag (a string of 6

Histidine amino acids that have a high affinity for nickel) selectively in order to allow for non-specific proteins to flow through (Bornhorst and Falke, 2000). Elution of N-PvdJp2 occurs when high imidazole is applied to the column, outcompeting N-PvdJp2 for binding sites and allowing the tagged protein to wash off the column in a single peak (Bornhorst and Falke, 2000). The fractions from this peak can then be combined and applied to a gel filtration column. Figure 3 illustrates the collection of N-PvdJp2 from fractions 47-65. The gel filtration column contains porous beads, which allow small molecules to diffuse slowly through the pores (Gel Filtration, n.d.). Larger molecules are not able to diffuse through the pores, and consequently move much quicker through the column. Gel filtration does not contain any denaturants, and therefore discriminates on the basis of molecular weight and 3-dimensional folding. Therefore, if dimers or tetramers of a protein of interest form, they will come out before the monomer, unlike during SDS-PAGE analysis (Gel Filtration, n.d.). Figure 4 shows that the last peak, sample 16, represents a monomer form of N-PvdJp2. Samples 15-17 were collected for concentration.

Buffer optimization screens were run on N-PvdJp2 to determine the optimum solubility for N-PvdJp2



On the Y-axis is Absorbance measured in mAU, and on the X-axis is volume measured in milliliters. My protein is indicated with the arrow and began eluting at 62 minutes.

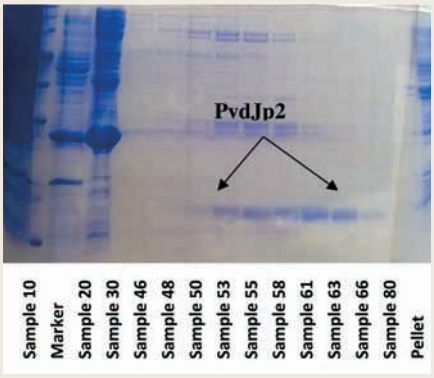
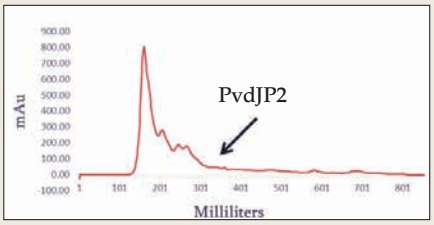


Fig. 3. Chromatogram and gel from Nickel Column



On the Y-axis is Absorbance measured in mAU and on the X-axis is volume measured in milliliters. My protein is indicated with an arrow and began eluting at 152 minutes.

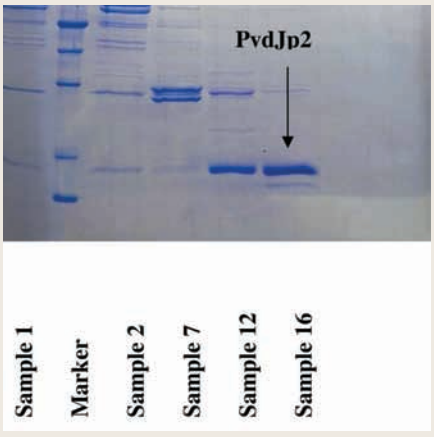


Fig. 4. Chromatogram and gel from Size Exclusion Column

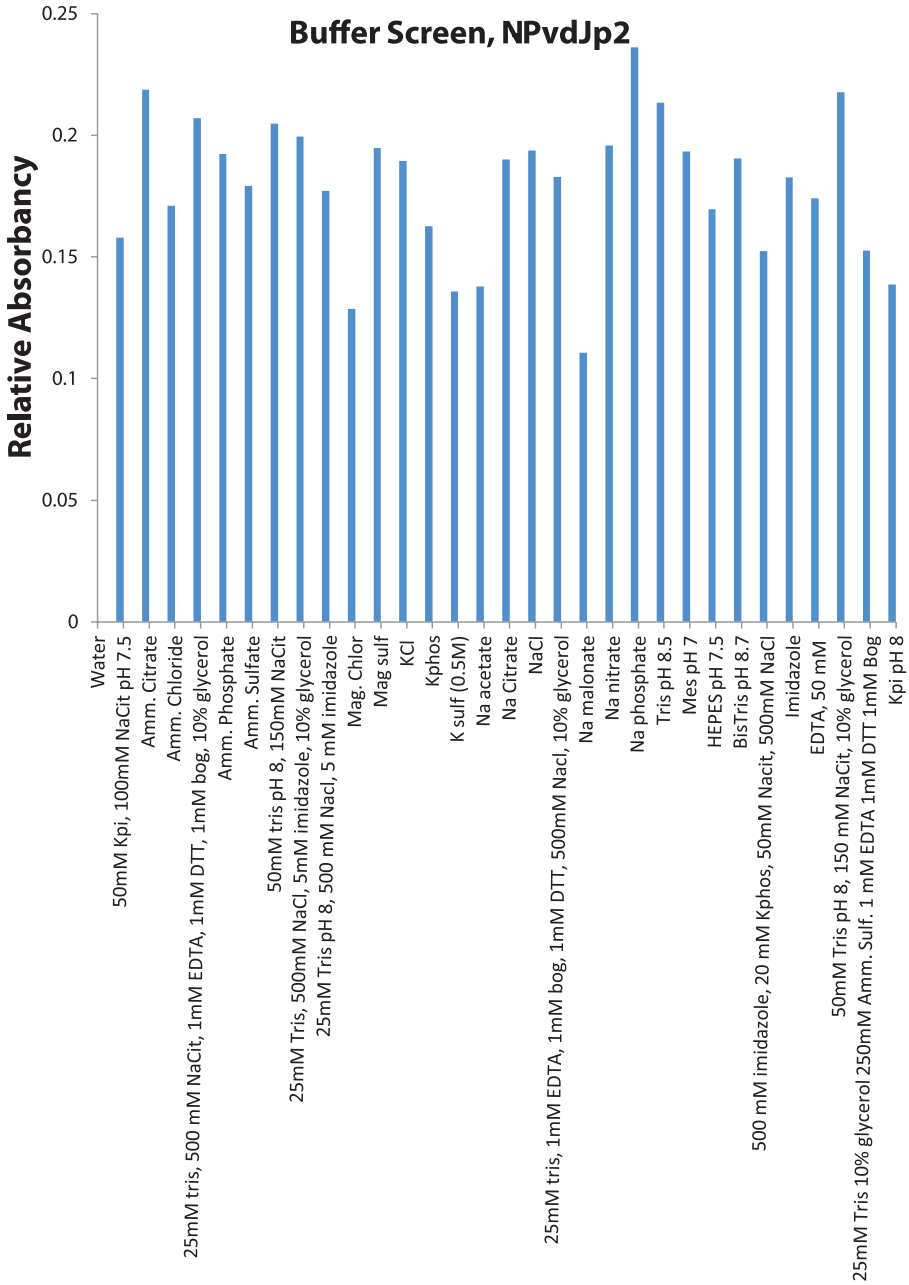


Table 3. Buffer optimization screen with N-PvdJp2. The addition of glycerol to Buffer A resulted in an increase in absorbance, and Buffer B was shown to be optimal for solubility.

in solution. When running a buffer screen, researchers are looking for high absorbency readings, indicating that the protein is soluble in solution and will therefore be present at higher concentrations when running crystal screen analysis. Results (pictured in Table 3) showed that in 25mM Tris pH 8, 5mM imidazole, 500mM NaCl, and 10% glycerol (Buffer A) solution N-PvdJp2 showed an absorbency of 0.199, comparable to the Buffer B solution of 50mM Tris pH 8, 150mM Sodium Citrate, and 10% glycerol at 0.218. N-PvdJp2 elutes at a concentration of 160mM Imidazole from the nickel chelating column. The resulting solution led to protein aggregates, most likely due to the high salt. Therefore, after being collected from the nickel chelating column, the protein was combined with equivalent amounts of Buffer B and concentrated with a stir cell. The addition of buffer before concentrating increased the protein collection significantly. After a complete preparation of Nickel and Gel filtration, 4mg of protein is prepared per liter of culture. The protein is determined to be 85% pure as estimated by coomassie gel. Protein was concentrated to 25mg/mL and stored at -80ºC until used in crystal screens.

Crystal trials were run with PvdJp2 in different conditions and concentrations. Trials included MCSG (Microlytic), Wizard (Emerald Biosciences) and Peg (Hampton Research). Crystal trials have thus far yielded no crystals suitable for X-ray analysis.

DISCUSSION

Great progress has been made in the characterization of PvdJp2. Optimum expression conditions and buffer screen analysis has led to a greater understanding of the purification process. In addition, crystal screens that do not yield diffraction-quality crystals have been ruled out from further study.

Once PvdJp2 has been characterized through X-Ray crystallography, it will provide further information about Peptidyl-Carrier Domains in NRPSs. As previously mentioned, NRPSs are presenting new options for antibiotic synthesis due to their ability to incorporate novel amino acids as well as the practical application of shifting the domains within a module to change the order of substrate incorporation (Doekel and Marahiel, 2000).

Determining the 3-dimensional structure of PvdJp2 is a small portion of the larger objective that the lab is trying to accomplish. PvdJ, as aforementioned, is a large, multi-enzyme complex composed of two full modules. Each of these modules incorporates a specific amino acid in a unidirectional fashion. The Lamb lab is currently working on several domains within PvdJ, including the full first module (PvdJm1), and the first PCP domain (PvdJp1). Once the 3-dimensional structures of each of these components have been determined, the lab can begin further work on analyzing where exactly the specificity for amino acid recognition comes from. Present research literature proposes mixed theories on whether it is the A domain or the C domain that confers specificity, as discussed below.

The Adenylation domain has been reported in many research studies as being responsible for recognition. In the article "Design and application of multi-modular synthetases," the authors report that the A domain is responsible for substrate recognition and adenylation driven by ATP consumption (Mootz and Marahiel, 1999). In another publication, the Adenylation domains are referred to as the "specificity conferring code" of NRPS systems (Stachelhaus et al., 1999). Ackerly and Lamont further this opinion, with the statement that A domains "provide active sites for the recognition and adenylation activation of a substrate amino acid" (2004).

A contrasting opinion is presented in the article "Construction of hybrid peptide synthetases," in which the authors state that while the C domain was not influential in selecting for specific amino acids, it is influential in "selectivity at the acceptor position of the C-domain of NRPSs for the correct stereoisomer" (Mootz et al., 2000). This selectivity shows a lower selectivity at the Donor site but a greater selectivity at the Acceptor site (Belshaw et al., 1999). This discovery indicates that the C domain is more selective towards the amino acids in the same module compared to amino acids in upstream modules (Lautru and Challis, 2004). In addition, low selectivity due to side chain size has also been observed (Ehmann et al., 2000).

A further goal for the overall PvdJ project would be to study the effect on swapping domains. Research has shown that domains can be swapped by fusion of linker regions and yield different products. Module fusions have been demonstrated to be increasingly effective, especially when the addition is within the linker region, where mutations, additions, deletions, and substitutions have no effect on the integrity of the NRPS (Doekel et al., 2008). Studies with Daptomycin have revealed efficiency of module exchanges exploiting the linker region to be as high as 50% (Nguyen et al., 2010).

Investigation of activity, specificity, and module or domain swapping will depend on the isolation and purification of each of the PvdJ domains. 3-dimensional modeling will provide additional insight into the functionality of these domains by providing a greater understanding of binding regions, conserved regions, and catalytic centers. In order to determine the structure of PvdJp2, further work must be done on crystal screen optimizations. Once determined, the structure can play a role in a greater understanding of NRPS systems and analysis.

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