SYMBIONT-PRODUCED BYROSTATINS: INVESTIGATION OF THEIR BIOSYNTHESIS AND EFFECTS ON HOST TARGET

Kayla I. Bean

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SYMBIONT-PRODUCED BYROSTATINS: INVESTIGATION OF THEIR
BIOSYNTHESIS AND EFFECTS ON HOST TARGET

by

KAYLA I. BEAN

Under the Direction of George E. Pierce, Ph.D.

ABSTRACT

The marine bryozoan *Bugula neritina* is the source of the bioactive polyketide compounds, the bryostatins. The biosynthesis of the bryostatins is putatively prescribed by the Type I modular polyketide synthase (PKS), *bry*, from the uncultured bacterial symbiont “Candidatus Endobugula sertula”. The *bry* gene cluster has a non-canonical, discrete acyltransferase (AT) enzymatic domain upstream of the PKS gene cluster. The AT is hypothesized to add the polyketide extender units onto an acyl carrier protein (ACP) in the *bry* PKS and, as such, is termed a *trans*-AT. In addition, some *trans*-ATs have been shown to have more extender unit substrate flexibility than *cis*-ATs, which are usually very substrate-specific. The ability of *trans*-ATs to discriminate between the modular ACPS and load them with the
correct extender unit to form the desired polyketide product during biosynthesis is unclear. To examine how \textit{trans-}AT’s discriminate between modules, protein-protein interactions between the BryP \textit{trans-}AT and interdomain regions between ketosynthase (KS) and AT regions of PKS modules were assessed using surface plasmon resonance (SPR) to compare binding events via dissociation data. On average, BryPAT\textsubscript{1} had a higher affinity for BryBM4 KS-AT interdomain region as compared to the EryAIII M5 KS-AT interdomain region.

Bryostatins are versatile compounds that are ecologically relevant for the survival of \textit{Bugula} larvae. The mechanism for this activity could be due to activation of protein kinase C (PKC) via high affinity for the PKC C1b domain. As the symbiont-produced bryostatins are potent activators of a eukaryotic cellular target, the question of how the bryozoan host has adapted to their presence arises. Interestingly, there is variation in symbiont and bryostatin status within the genus \textit{Bugula}, with some species possessing a symbiont that produces bryostatins, some species possessing a closely-related symbiont that does not produce bryostatins, and some species with no symbiont. Using SPR, on average, bryostatin has a higher affinity for bryostatin-producing \textit{B. neritina} C1b domain as compared to non-bryostatin containing \textit{Bugula pacifica} and \textit{Rattus norvegicus} C1b domains. Understanding aspects of bryostatin biosynthesis and symbiont effects on the host will provide deeper insight into the vital role that they play in the interaction between \textit{B. neritina} and \textit{E. sertula}.

INDEX WORDS: Symbiosis, Bryostatins, Protein kinase C, Polyketide synthesis, gene expression, natural product-protein interaction
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KAYLA I. BEAN

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December 2017
DEDICATION

I would like to dedicate my doctorate to my family. My family has had nothing but support for me throughout my education and during the long trying time at Georgia State University. To my parents, Jimmy and Nancy Bean, who have always believed in me and have had the faith that I could accomplish anything I wanted to. My sister, Crystal, for all the time she took to help me over the years whether it was driving me to any one of my many sports games or paying my cell phone bill. To my sister, Melanie, who gave me the encouragement, strength, and financial support to get through all my education programs. If Mel had not told me to stick with it and to not let myself get weeded out I wouldn’t be where I am today. I would like to thank all my aunts, uncles, and cousins for their support and pride in me. To Chris Newsom, thank you for all the love and support you have given me during our relationship. You truly have made me a better person and have been my rock. I don’t know how I would get through life without you. I would like to thank the lifelong friends that I made during my time at the University at Tennessee, Holly, Justin, and Brandon. Thank you for being best friends over the years and always standing by me. Your friendship has meant so much to me; you truly are family to me. To my CDC colleague and my biggest supporter, Dr. Marty K. Soehnlen, I am so proud to call you my friend. Your belief and encouragement over our friendship has made such a difference in my life. Thank you to Colin MacLean, I am very privileged to have your friendship; you are someone that I will always be able to count on. I would like to thank all my family and friends who have made such an impact in my life; I will never be able to fully express the gratitude I have for everyone who has helped or shaped me along the way.
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1 BACKGROUND

1.1 Natural Products and Symbiosis

Natural products are small molecules that possess biological and/or pharmaceutical activities produced by organisms ranging from microorganisms to plants to animals (Li & Vederas, 2009). Many bioactive products are formed as a result of symbiotic interactions (Piel, 2009), associations between a host and symbiont with a range of outcomes, from mutually beneficial to parasitic. These relationships can be obligate or facultative, and have a major impact on the survival and co-evolution of the partners (Moran, 2006). In microbial symbiotic relationships, natural products can be utilized to aid in host development or defense (McFall-Ngai et al., 2013). For instance, an entomopathogenic nematode has a mutualistic relationship with the insect pathogen *Photorhabdus luminescens* that produces the antimicrobial compound hydroxystilbene, which aids in the growth of juvenile nematodes (Strauch & Ehlers, 1998, Joyce et al., 2008). Symbiont-produced bioactive compounds can also play a role in host defenses against a predator or parasite (Haine, 2008, Lopanik, 2014). The bacterial symbiont found in the female rove beetle, *Paederus*, produces the compound, pederin, which protects eggs and larvae of the beetle from predatory spiders (Kellner & Dettner, 1996). Many bioactive symbiont-produced natural products have potential therapeutic applications. The parasitic fungus *Rhizopus* sp. has an endosymbiont, *Burkholderia* sp. that produces the anti-cancer compound rhizoxin (McLeod et al., 1996, Partida-Martinez et al., 2007, Schmitt et al., 2008), which causes antimitotic activity by binding to tubulin (Tsuruo et al., 1986, McLeod et al., 1996, Scherlach et al., 2006). The fungal symbiont of the jellyfish *Nemopilema nomurai* produces four polyketides with antibacterial activity against pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus* 3089 (Liu et al., 2011). The antitumor compounds mycalamides,
onamides, and theopederins, similar to pederin, but isolated from different marine sponges, have the ability to inhibit protein synthesis and cell division (Piel et al., 2004, Witczak et al., 2012). These examples illustrate the importance of symbiont-produced natural products in symbiotic relationships, and their bioactivity.

### 1.2 Bryostatins

Another example of symbiont-produced natural products is the bryostatins (Figure 1.1). The marine bryozoan *Bugula neritina* is the source of these 20 polyketide metabolites (Pettit et al., 1982, Pettit, 1996, Lopanik et al., 2004). *B. neritina* harbors the uncultured endosymbiotic γ-proteobacterium, “*Candidatus Endobugula sertula*” (Haygood & Davidson, 1997), which is thought to biosynthesize the bryostatins (Davidson et al., 2001, Lopanik et al., 2004, Sudek et al. 2012).

![Figure 1.1: Structures of bryostatins, with substituent groups at R₁ and R₂ indicated in table. Bryostatins 3, 19, and 20 feature cyclization indicated with asterisks (*). (Adapted from Davidson and Haygood, 1999.)](image)
Some bryostatins are unpalatable, and defend *B. neritina* larvae from predators (Lindquist, 1996, Lindquist & Hay, 1996, Lopanik *et al.*, 2004). In eukaryotic cells, bryostatins activate protein kinase Cs (PKC) (Wender *et al.*, 1988) are serine-threonine kinases involved cell signaling cascades of many regulatory processes such as cell growth and structure (Newton, 2001, Newton, 2010).

### 1.3 *Bugula neritina*

The marine bryozoan *Bugula neritina* is a colonial invertebrate found world-wide in temperate habitats. A bryozoan colony is composed of individuals called zooids. In *B. neritina*, embryos are brooded in chambers, termed ovicells, found on the maternal zooid, where they develop into larvae. Once developed, the soft bodied, nutrient-rich *B. neritina* larvae are released into the water column (Woollacott & Zimmer, 1972) where they are vulnerable to predation but are protected by the presence of the distasteful bryostatins (Lindquist, 1996, Lindquist & Hay, 1996, Lopanik *et al.*, 2004). In larvae, the endosymbiont *E. sertula* resides within an indentation on the surface known as the pallial sinus (Woollacott, 1981, Haygood & Davidson, 1997). In adult colonies, the symbiont is located in the funicular cords (Woollacott & Zimmer, 1975, Sharp *et al.*, 2007), which act as a vascular system within the colony, transporting nutrients and waste (Carle & Ruppert, 1983). Funicular cords also act as a placental system, providing nutrients to the growing embryo in the ovicell; it is hypothesized that the symbiont is transmitted to the next generation larvae through the funicular cords (Woollacott & Zimmer, 1975, Sharp *et al.*, 2007).

Symbiosis is not always characteristic of *B. neritina* or other *Bugula* spp. Both *B. neritina* and *B. simplex* have been shown to possess closely-related, but different species of bryostatin-producing symbiont (Davidson & Haygood, 1999, Lim & Haygood, 2004), while in
contrast other species (*B. stolonifera* and *B. turrita*) lack a symbiont (Woollacott, 1981, Lim-Fong *et al.*, 2008). In another variation, *B. pacifica* and *B. turbinata* are thought to possess a symbiont but no bryostatins (Lim-Fong *et al.*, 2008). Furthermore, *B. neritina* displays variability in symbiosis with *E. sertula* with both symbiotic and aposymbiotic colonies of *B. neritina* having been identified. Currently, three sibling species have been identified using *B. neritina* mitochondrial cytochrome *c* oxidase *I (COI)* sequences: Type D, Type S, and Type N (Davidson & Haygood, 1999, McGovern & Hellberg, 2003, Fehlauer-Ale *et al.*, 2014). In the United States, Type S and Type D have been found in southern California occurring above the depth of 9 m and a depth below 9 m, respectively, and display differences of 8.1% in a region of the *COI* gene. Interestingly, the symbiont within each host sibling species differ 0.4% their 16S rRNA genes, and bryostatin composition also varies (Davidson & Haygood, 1999). The Type D contains chemotype O bryostatins (bryostatins 1-3), that possess an octa-2, 4-dienoate ester at C20 of the bryostatin pyran ring (Figure 1). Bryostatins found in Type S colonies do not contain the octa-2, 4-dienoate ester, and were designated chemotype M. Differences in bryostatin chemotypes are due to the variation in the *bry* gene cluster expression and composition (Sudek *et al.*, 2007). On the Atlantic coast, *B. neritina* found north of Cape Hatteras have historically been identified as Type N while colonies found south of the Cape Hatteras and in the Gulf of Mexico have been characterized as Type S genotype (Davidson & Haygood, 1999, McGovern & Hellberg, 2003). Populations north of Cape Hatteras, Type N, have a divergence of 11.5% from the Type S *COI* sequences. Interestingly, this haplotype was found not to possess the symbiont (McGovern & Hellberg, 2003), or unpalatable bryostatins (Lopanik *et al.*, 2004). It has been proposed that less predation pressure at higher latitudes results in reduced defenses in organisms
at higher latitudes (Vermeij, 1978, Bertness et al., 1981, Menge & Lubchenco, 1981), and the occurrence of undefended, aposymbiotic colonies supported this hypothesis.

More in depth studies of the population structure of host and symbiont have shown that Type S and Type N can be found on either side of the Cape Hatteras and in some cases present in the same locations. Further, colonies of both Type S and N sibling species have been found to be symbiotic as well as aposymbiotic. These data indicate that the bryostatins may not be as crucial for host defense as past research has shown (Linneman et al., 2014). If the presence of the symbiont-produced bryostatins is not solely for the purpose of defense, then they may be required for other functions within the host. Also not all symbiotic Bugula spp. possess bryostatins, as in the case of B. pacifica and B. turbinata (Lim-Fong et al., 2008), suggesting that the relationship is not completely reliant on the bryostatins. This leads to the question of why do some species have the bryostatins and others do not. Also, how do the bryostatins effect the host when present? It is possible the host has adapted to the presence of symbionts and symbiont-producing bryostatins over time, which has led to a co-evolution event between host and symbiont.

1.4 Bugula species

*Bugula* species are a diverse group of bryozoans with varying morphologies, symbionts, and environments. Bugula species can be found globally in the oceans whether cold or warm waters (Brock, 2017). Most species are found in fouling habitats and shallow waters; however, there are bryozoan communities found in deeper regions of the ocean (Davidson & Haygood, 1999, McGovern & Hellberg, 2003, Fehlauer-Ale et al., 2014). While *B. neritina* has been widely studied, other *Bugula* species such as *Bugula stolonifera, Bugula pacifica, Bugula turrita, Bugula turbinata,* and *Bugula simplex* have unique characteristics that are of interest. While *B.
neritina colonies have a distinct purple pigmentation, B. stoloniferia and B. turrita are white to grey in color whereas B. turbinata and B. simplex’s tint can range from brown to yellow colonies (Hincks, 1886). B. simplex have multi-serial branching, hemispherical ovicells, and long avicularia only on marginal zooids. B. stolonifera colonies have bi-serial branching with sub-globular ovicells and avicularia. B. turrita has bi-serial, spiral branching with avicularia along outer lateral of zooid with caduceus oviceells (Ryland, 1960, Ryland, 1991, Ryland et al., 2011). B. turbinata have multi-serial branching with avicularia. Extracts from B. pacifica, B. turbinata, B. turrita, B. stolonifera have shown no bryostatin activity while B. neritina and B. simplex have bryostatin activity (Lim & Haygood, 2004). The contrast between Bugula spp. of symbiont with bryostatins, symbiont with no bryostatins, and no symbiont, no bryostatin could provide natural controls for research on the symbiont’s effect on host.

1.5 Bryostatin Biosynthesis

Bryostatins are polyketide molecules that are typically biosynthesized by modular polyketide synthases (PKS). Type I PKSs are large modular protein complexes that synthesize polyketides in a linear assembly-line fashion, with each enzyme being used only once in the biosynthesis [reviewed in (Fischbach & Walsh, 2006)]. Type I PKSs are organized in modules, with each module containing multiple catalytic domains responsible for one elongation and modification of the nascent polyketide. The enzymatic domains responsible for polyketide elongation are acyltransferase (AT), acyl carrier protein (ACP), and ketosynthase (KS) while other enzymatic domains, such as the ketoreductase (KR), dehydratase (DH), enoylreductase (ER), methyltransferase (MT), and thioesterase (TE) domains, modify the polyketide chain (Staunton & Weissman, 2001, Fischbach & Walsh, 2006). During elongation, monomer units, usually malonyl-CoA or methylmalonyl-CoA, are used to extend the nascent polyketide chain by
two carbons (Haydock *et al*., 1995, Keatinge-Clay *et al*., 2003). For elongation in typical Type I PKSs, the dedicated AT domain selects a specific extender unit and loads the unit onto its active site serine. The AT then transfers the extender unit covalently to the activated ACP. Research to date suggest that AT domains exhibit a high level of substrate specificity in regard to the selection of the extender unit, and certain AT amino acid residues are indicative of the substrate (Haydock *et al*., 1995, Keatinge-Clay *et al*., 2003). The KS catalyzes the condensation reaction of the ACP-bound extender unit to the nascent polyketide chain (Staunton & Weissman, 2001).

Polyketides such as erythromycin and pikromycin are formed in this general pattern of elongation. Erythromycin is a 14-membered macrolide antibiotic (Woodward *et al*., 1981) produced by the actinomycete *Saccharopolyspora erythraea* (McGuire *et al*., 1952, Cortes *et al*., 1990, Staunton & Wilkinson, 1997). The preferred substrate for extender units for all of the AT domains within the erythromycin PKS (DEBS) is methylmalonyl-CoA (Donadio *et al*., 1991).

Pikromycin is a 14-membered ring macrolide antibiotic produced by another actinomycete, *Streptomyces venezuelae*. The AT domains within the pikromycin PKS cluster (Pik) utilize malonyl-CoA and methylmalonyl-CoA as extender units in different places (Xue *et al*., 1998).

### 1.6 Acyltransferases in Bryostatin Biosynthesis

The bryostatin PKS (*bry*) is different from canonical Type I modular PKSs as each module lacks embedded AT domains, and, instead, has AT domains on an ORF upstream of the modular PKS genes (Sudek *et al*., 2007). There are other PKS systems that have discrete AT domains, such as the leinamycin (Cheng *et al*., 2003), bacillaene (Butcher *et al*., 2007), pederin (Piel,
Figure 1.2: Domain architecture of Bry, Ery, and Pik PKS modules with native substrates. (A) trans-AT BryBM4 with malonyl-CoA, (B) cis-AT Ery AIII M6 with methylmalonyl-CoA, and (C) cis-AT PikAIV M6 with methylmalonyl-CoA.

Phylogenetic analysis indicated that BryPAT1 is closely related to other trans-ATs that utilize malonyl-CoA as a substrate, and in vitro biochemical analysis demonstrated that it loaded malonyl-CoA onto variety of excised ACPs (Lopanik et al., 2008). BryP AT1 loaded both clusters. LmnG, a discrete AT from the leinamycin gene cluster, was shown to load extender units onto ACPs and an incomplete module in vitro (Cheng et al., 2003). In bry, the two AT domains BryPAT1 and BryPAT2 are not embedded within the modules, but instead are located upstream of the modular PKS genes in a single ORF called BryP. The discrete nature of the AT domains suggests a trans mode of action, in which one AT, BryP, loads extender units on each modular ACP within the PKS (Sudek et al., 2007) (Figure 1.2).
malonyl- and methylmalonyl-CoA onto two excised ACPs from the bry gene cluster (BryB M7 ACP and BryB M3 ACP), although it prefers malonyl-CoA. Bryostatins have two geminal dimethyl groups (C8 and C18, Figure 1.1) that are hypothesized to be produced by modules with methyltransferase (MT) domains (BryBM4 and BryCM9) (Sudek et al., 2007). These modules could utilize either methylmalonyl-CoA or malonyl-CoA, and the MT would methylate the nascent polyketide chain either once (with methylmalonyl-CoA) or twice (with malonyl-CoA).

As previously mentioned, the erythromycin gene cluster (Ery) has cis-ATs with a native substrate of methylmalonyl-CoA for all modules, while pikromycin (Pik) cis-AT native substrates are methylmalonyl-CoA (for modules 1-4, 6), and malonyl-CoA (module 5) (Figure 1.3: PKS module loading using $[^{14}C]$ malonyl-CoA and $[^{14}C]$ methylmalonyl-CoA. (A) BryPAT$_1$ is able to transfer malonyl-CoA onto EryAIII M6 AT$^\circ$ and holo BryB M4. (B) BryPAT$_1$ is unable to transfer malonyl-CoA or methylmalonyl-CoA on PikAIV M6 AT$^\circ$ (null-AT). PikAIV M6 wild-type (wt) is able to self-load native methylmalonyl-CoA. From (Lopanik, et al., 2008).
Further experiments with overexpressed complete modules demonstrated that BryP AT$_1$ was able to complement the EryAIII M6 AT$^o$ mutant by loading malonyl-CoA (the unnatural substrate) onto the cognate ACP (Figure 1.3A). Interestingly, BryP AT$_1$ was unable to load malonyl-CoA onto the AT-null mutant module 6 of the pikromycin gene cluster (Figure 1.3B); the PikAIV M6 also utilizes methylmalonyl-CoA as its native substrate. The inability of BryP AT$_1$ to load a substrate onto PikAIV M6 AT$^o$ despite the similarity of the domain composition of both modules (EryAIII M6: KS, AT, KR, ACP, TE, and PikAIV M6: KS, AT, ACP, TE) and native AT substrates (methylmalonyl-CoA), indicates a possible recognition system by which the trans-AT can discriminate between the modules.

1.7 Host Adaptation to Natural Products

Microbes are a prolific source of bioactive natural products, many of which have been developed into pharmaceuticals (Li & Vederas, 2009). Microbes that are in mutualistic relationships with eukaryotic hosts can also produce bioactive compounds. In some cases, these compounds can play a role in the defense of the host (Moran, 2006). For example, pederin is a symbiont-produced compound that allows rove beetle larvae to gain protection for predatory wolf spiders (Piel, 2002). *Enterococcus faecalis* has been shown to its protect host, *Caenorhabditis elegans*, from other bacterial pathogens such as *Staphylococcus aureus* by increasing antimicrobial superoxide production (King *et al.*, 2016). *Actinobacteria* strains found in insects like bees and wasps may produce secondary metabolites that confer protection against natural predators (Abt *et al.*, 2012). However, most of the time, the symbiont’s role in the relationship with the host has not been elucidated. While there are several examples to illustrate the external effects that symbiont produced compounds can have on the host, internal effects may be much harder to confirm. The close association of host and symbiont, by nature, means
that symbiont-produced bioactive compounds may interact with eukaryotic host cellular targets to elicit change in the host. The extent of host adaptation to the presence of these compounds is not well understood for most relationships. One exception is the relationship between the plant-pathogenic fungus *Rhizopus microsporus* and its bacterial symbiont, *Burkholderia rhizoxinica* (Partida-Martinez & Hertweck, 2005). Fungal strains that contain the rhizoxin-producing symbiont possess a mutation in their β-tubulin gene. The mutation allows for a decreased affinity to rhizoxin, an otherwise toxic compound (Schmitt *et al.*, 2008). This relationship demonstrates significant co-evolution between host and symbiont; it is also possible that host and symbiont coevolve such that these compounds can become important for normal host physiology.

1.8 **Protein Kinase C and Bryostatin**

Protein kinase C (PKC) is a serine-threonine kinase involved in transducing cellular signals (Newton, 1995, Newton, 2001). PKCs are important in fundamental processes such as cell

![Figure 1.4: Architecture of PKC isoform domains.](image)

cPKC = conventional, nPKC = novel PKC, and aPKC = atypical PKC. PS = phosphatidyl serine, DAG = diacyl glycerol. (Adapted from Newton, 2001 and Rosse, *et al.*, 2010)
growth, changes in cell pH levels, and cytoskeleton structure alterations (Newton, 1995, Griner & Kazanietz, 2007). When PKCs are inactive, they are found in the cytosol of the cell (Newton, 2001); upon activation, PKCs travel to the cell membrane for cell signaling and are eventually degraded by proteases (Newton, 1995). There are three types of PKCs isoforms: conventional (cPKC, α, βI, βII, γ), novel (nPKC, δ, ε, η, θ), and atypical (aPKC, ξ, λ, ι) (Figure 1.4).

All three isoforms possess a catalytic domain, but differ in their regulatory domains (Newton, 2001, Newton, 2010). The cPKCs have C1a and C1b Zn-finger domains that bind DAG, a pseudosubstrate domain, and a calcium binding domain. The nPKCs differ by not having a calcium binding region. The aPKCs lack the calcium binding domain and the C1b region of the DAG-binding domain (Newton, 2001).

Bryostatin binds with high affinity to the C1b domains in cPKCs and nPKCs (Kraft et al., 1986, De Vries et al., 1988, Kraft et al., 1988). Bryostatin 1 has been shown to induce rapid

![Figure 1.5: Translocation of PKCβ1-GFP by 200 nM bryostatin 1 in transiently transfected CHO-k1 cells.](image)

(A) predose, (B) 5 min postdose, and (C) 38 min postdose (D–F) translocation of PKCδ-GFP by 200 nM bryostatin 1, same time points (Wender, et al., 2011)
translocation in δPKC and βPKC to the cell membrane in transfected CHO cells whereas bryostatin 2, differing by a C7 acetate group, has no effect (Figure 1.5). Conventional αPKC was also activated but to a lesser degree. Furthermore, bryostatin 1 had a higher level of PKCα degradation after prolonged activation as compared to PKCδ and PKCβ (Wender et al., 2011). Differing activation levels and selectivity of the bryostatins among PKC isoforms suggests that these compounds could regulate PKC.

1.9 Bryostatin effects on B. neritina PKCs

Microbial endosymbionts are often the source of bioactive natural products, and in order to fully understand host/symbiont coevolution, it is necessary to understand the effects of these compounds on the host. It is reasonable to propose that, due to the close association between B. neritina and E. sertula, and the high affinity of the bryostatins for eukaryotic PKCs, the presence of the bryostatins has influenced the evolution of the host PKCs. In B. neritina, extended exposure to bioactive bryostatin metabolites has likely led to host adaptation to the symbiont and/or symbiont metabolites (Mathew et al., 2016). Over time, the host could adapt to the presence of the symbiont-produced bryostatins such that they are have become necessary for host physiology.

In order to identify potential effects of the symbiont on the host, the symbiont was cured from B. neritina using an antibiotic treatment to observe colonies without the symbiont. Colony growth was similar in control and cured colonies, indicating that the presences of the symbiont and bryostatins had no significant effect on growth. Interestingly, significantly fewer reproductive structures, ovicells, were observed in B. neritina symbiont-reduced colonies (Mathew et al., 2016). This suggests that either the symbiont or the symbiont-produced bryostatins may be involved in the physiological process of reproduction. In addition, Western
blot analysis of bryostatin-activated cPKCs in symbiotic colonies exhibited lower molecular weight proteins in total protein extracts (Mathew et al., 2016). Crude protein extracts from antibiotic treated *B. neritina* colonies, however, did not have the same lower molecular weight banding pattern. Similar results were also seen in protein extracts of additional naturally symbiotic and aposymbiotic colonies (Mathew et al., 2016). These results indicate that PKCs in *B. neritina* are potentially affected by the presence of bryostatins. As previously mentioned, bryozoans in the genus *Bugula* vary in their symbiotic state, with some species being aposymbiotic, and some host species possess a symbiont closely-related to *E. sertula* that does not produce bryostatins (Lim-Fong et al., 2008). These varying species present natural controls to test the hypothesis that the host PKC has evolved in the presence symbiont-produced natural products.

1.10 Research Aims

The *in vitro* protein-protein interactions between heterologously expressed BryPAT1 and KS-AT interdomain regions of BryB M4, EryAIII M6, and Pik AIV M6 is one goal of this research. Binding affinities of possible protein interactions will be determined using surface plasmon resonance. Further work to elicit information about specific amino acids involved in the proteins ability to bind the *trans*-AT will be studied by amino acid substitutions using site-directed mutagenesis. Changes in the amino acid sequences could change the ability of BryPAT1 to bind.

Understanding how bryostatin biosynthesis occurs is important; nonetheless, identifying affects bryostatins could have on the host is also vital. Since bryostatins have the ability to activate PKCs, another goal of research will focus on identifying PKC C1b domain in *B. neritina* and *Bugula* spp. Once C1b domains are detected, each sequence will be cloned into a vector for
protein expression. The C1b domain constructs from the multiple species will be tested against bryostatin-1 using SPR to determine binding affinities and possibly pinpoint amino acid residues involved in the PKC activation.
2 MODULE RECOGNITION BY DISCRETE ACYLTRANSFERASE (AT) IN BRYOSTATIN BIOSYNTHESIS

2.1 Introduction

Polyketide synthases are large, multi-modular proteins that catalyze the biosynthesis of polyketide molecules (Keatinge-Clay, 2012). The nascent polyketide chain is covalently attached to the PKS, and is essentially “passed along” the enzyme as it is grown and modified. Often, the PKS modules are divided on different ORFs, such that the growing polyketide chain will have to be transferred from a module on one ORF to a module on the next ORF. Because of this, protein-protein recognition is important for many aspects of polyketide biosynthesis such as module to module interaction, delivery of the substrate to the ACP, and in catalytic steps involving the KS domains [reviewed in (Xu et al., 2013)]. In PKS gene clusters with trans-AT domains, the free-standing AT must be able to load the extender unit onto the cis-ACP domain. Further, some PKSs have more than one trans-AT domain, with different extender unit substrate preferences, which must load the correct cis-ACP domain with the correct extender unit (Musiol et al., 2011). This indicates that trans-AT domains have the ability to recognize their cognate embedded ACP domains. The trans-AT has been identified in several natural product-bacterial systems from exotic prokaryotes to pathogenic bacteria as well as those involved in biocontrol (Piel, 2010). Pederin (Piel, 2002), kirromycin (Weber et al., 2008), Rhizoxins (Partida Martinez & Hertweck, 2005, Loper et al., 2008) are all bacterial natural products synthesized by PKS systems with trans-ATs.

Previous data suggest that the trans-AT BryP AT$_1$ can discriminate between modules, as it was able to load malonyl-CoA onto BryB M4 and EryAIII M6, but not on PikAIV M6.
One mechanism that may account for this differential activity is protein-protein recognition of the KS-AT interdomain regions. In order to test the hypothesis that KS-AT interdomain regions are important for trans-AT recognition, sequences of BryPAT_1 and the Ery, Pik, and Bry KS-AT interdomain regions were identified, cloned into an expression vector, and overexpressed. Overexpressed proteins were purified and concentrated to allow for in vitro protein-protein interactions quantification using surface plasmon resonance (SPR). In SPR, an optical biosensor detects changes in the binding of an immobilized molecule with an analyte molecule in real-time without labeling (Malmqvist, 1999). Changes in light reflection in response to a binding event plotted against time provides information about molecule interactions and compound affinities. A range of SPR experiments can be achieved using chips that can consist of a gold side for electron excitation and another side that can be composed of various dextran matrixes to immobilize molecules. Each dextran matrix is geared toward a specific molecule immobilization and experiment (Englebienne et al., 2003, Zeng et al., 2014). For example, histidine-tagged proteins can be attached to a nitrilotriacetic acid (NTA) dextran sensor chip via nickel II solution which can be regenerated (Kimple et al., 2010). CM5 sensor chips have a carboxymethylated dextran that irreversibly immobilize molecules via -NH_2, -SH, -CHO, -OH, or -COOH groups (Drescher et al., 2009). In this study, levels of interaction will be based upon binding affinities of the BryPAT_1 to the KS-AT interdomain regions of BryB M4, EryAIII M6, and PikAIV M6. I hypothesize that the binding affinities for BryPAT_1 to BryB M4 KS-AT and EryAIII M6 KS-AT will be higher than in Pik AIV M6 KS-AT. Binding affinities of BryPAT_1 with the KS-AT interdomain regions will confirm if the trans-AT can recognize and interact within these regions of the module for extending nascent polyketide chains.
2.2 Materials and Methods

2.2.1 Cloning of KS-AT interdomain regions in BryBM4 and PikAIVM6

2.2.1.1 KS-AT interdomain region identification and primer design

The KS-AT interdomain regions of BryB M4 (ABM63527), PikAIV M6 (AF079138), and EryAIII M6 (Q03133) modules were located by identifying the KS and AT domains using MotifScan. Amino acid alignments of other AT-less PKS systems (Tang et al., 2004) were used to identify conserved amino acid residues to determine the 3’ end of the KS-AT regions in BryB M4, as the lack of AT domain made the C-terminus more difficult to identify. GOR software (Garnier et al., 1996) was used to identify ordered regions (i.e., α-helix, β-sheet), and constructs were designed so that the N- and C-termini occurred on a non-ordered, extended region. Primers were designed to flank the KS-AT interdomain regions in extender regions as to not disrupt areas of α helices or β sheets.

2.2.1.2 PCR of KS-AT interdomain regions

Multiple primer sets (Table 2.1) were designed to amplify KS-AT interdomain regions for BryBM4, Ery AIII M6, and PikAIV M6. The KS-AT interdomain region of BryBM4 was PCR amplified using Phusion High-Fidelity DNA polymerases (Thermo Scientific, Pittsburgh, PA, USA) protocol. PCR reactions were carried out by initial denature at 98°C for 30 s, with 35 cycles of denatured at 98°C for 10 s, annealed for 30 s (temperature varied per primer set), and elongated at 72°C for 25 s, and final extension of 72°C for 5 min. PCR amplicons were separated using gel electrophoresis. PCR amplicons of the correct size were then purified using GeneJET PCR purification kit (Thermo Scientific, Vilnius, Lithuania).
Table 2.1 Primers used in KS-AT Cloning

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Target</th>
<th>Product size (bp)</th>
<th>Annealing Temperature</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>BryKS-AT F</td>
<td>TGAGTCAATTCAAATGTTATCAGTAGAT</td>
<td>BryBM4 KS-AT Region</td>
<td>978</td>
<td>47°C</td>
<td>PCR</td>
</tr>
<tr>
<td>BryKS-AT R</td>
<td>ATAAAACCTCGAGCGTACTCCATTGCTCAGG</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EryKS-AT F</td>
<td>CCTGTTGAATTCTGCTTCGCG</td>
<td>Ery AIII M6 KS-AT Region</td>
<td>490</td>
<td>64°C</td>
<td>PCR</td>
</tr>
<tr>
<td>EryKS-AT R</td>
<td>ACCCTGCTCGAGGAATCAGAAAAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PikKS-AT F1</td>
<td>CTGCGGAGCTCGAGAACGTGACGTGACGT</td>
<td>PikM6 KS-AT Region</td>
<td>482 691 823</td>
<td>60°C 60°C 60°C</td>
<td>PCR</td>
</tr>
<tr>
<td>PikKS-AT F2</td>
<td>GAGCGACGCGACTGTCGGACGTGCGGTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PikKS-AT F3</td>
<td>CACCAGGACTCGGTCCCGCTCGCGCTCGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PikKS-AT R1</td>
<td>GCCGGGCGAGCTGACGTGACGTGACGTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td>TAATACGACTCACTATAGGG</td>
<td>T7 Promoter Region</td>
<td>343 (+insert)</td>
<td>54°C</td>
<td>PCR</td>
</tr>
<tr>
<td>T7 Terminus</td>
<td>GCTAGTTATTGTCACGCGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.1.3 Synthetic PikAIV M6 Construction

After several unsuccessful attempts to amplify and clone the PikAIV M6 KS-AT region, the KS-AT interdomain region was synthetically constructed using gBlocks® Gene Fragments (IDT, Coralville, IA, USA). The theoretical sequence was manually codon optimized with inserted restriction digest sites for SacI and a stop codon just before a XhoI site. SacI and XhoI (NEB, Ipswich, MA, USA) were used to digest synthetic PikAIV M6 KS-AT region for 2 hours at 37°C. The digested gene fragment was then visualized using gel electrophoresis to ensure proper DNA size. The DNA band was purified using GeneJET gel extraction kit (Thermo Scientific, Vilnius, Lithuania) and ligated into a SacI and XhoI digested pCDF-1b vector (Novagen) containing an N-terminal 6x Histidine tag using T4 Ligase (NEB, Ipswich, MA, USA) for 4 hours at 16°C. The ligated products were transformed into E. coli XL1-Blue (Stratagene) chemically competent cells (E. coli Transformation Buffer set, Zymo Research
Corp., Irvine, CA, USA), plated on Luria Bertani- Miller agar (LB) (BD, Sparks, Maryland, USA) with 50 μg/mL kanamycin (KAN), and incubated at 37°C overnight. Resulting colonies were screened for insert by colony PCR using T7 and T7 terminus primers (Table 2.1) and One Taq Polymerase (NEB, Ipswich, MA, USA). PCR reactions were carried out by initial denature at 94°C for 5 minutes, with 35 cycles of denatured at 94°C for 30 sec, annealed for 30 sec at 54°C, and elongated at 68°C for 50 sec, and final extension of 68°C for 5 min. Colony PCR amplicons of pCDF-1b::PikAIV KS-AT with the size of interest were extracted using Zyppy™ Plasmid Miniprep Kit (Zymo Research Corp., Irvine, CA, USA).

2.2.1.4 Construction of Recombinant Plasmids

EA011 was previously constructed by ligating a PCR amplicon [generated with Phusion Taq (NEB, Ipswich, MA, USA)] into the pET28 vector via EcoRI and XhoI digest sites. The BryBM4 PCR amplicons were digested with EcoRI and XhoI for 2 hours at 37°C and visualized using gel electrophoresis to ensure proper DNA size. Digest DNA band was excised and purified using GeneJET gel extraction kit (Thermo Scientific, Vilnius, Lithuania). BryBM4 purified, digested product was then ligated into a purified EcoRI and XhoI digested pET28b vector (Novagen) containing an N-terminal 6x Histidine tag using T4 Ligase (NEB, Ipswich, MA, USA) for 4 hours at 16°C. The ligated products of BryB M4 and Ery AIII M6 were transformed into chemically competent E. coli XL1-Blue (Stratagene) chemically competent cells, plated on LB agar with 50 μg/mL kanamycin (KAN), and incubated at 37°C overnight. Resulting colonies were screened for insert by colony PCR using T7 and T7 terminus primers (Table 2.1) and One Taq Polymerase (NEB, Ipswich, MA, USA). PCR reactions were carried out by initial denature at 94°C for 5 min, with 35 cycles of denatured at 94°C for 30 s, annealed at 54°C for 30 s, and elongated at 68°C for 50s, and final extension of 68°C for 5 min. Colony PCR
amplicons of pET28b::BryBM4 KS-AT were visualized using gel electrophoresis and bands with the size of interest were extracted using Zyppy™ Plasmid Miniprep Kit (Zymo Research Corp., Irvine, CA, USA) and quantified using Nandrop.

After plasmid purification, the insert was sequenced using the T7 and T7 terminator primers at the DNA Analysis Facility at Yale University (New Haven, CT, USA). Consensus sequences were aligned to theoretical construct sequences for confirmation using MegAlign (Lasergene) software. Colonies with the correct insert were named Ery AIII M6 (EA011), BryBM4 KS-AT (pKB114), and PikAIV M6 (pKB027).

2.2.2 Protein Overexpression of KS-AT Recombinant Constructs

2.2.2.1 Cell Growth and Induction

BryPAT1 (pNL020), BryBM4 KS-AT (pKB114), Ery AIII M6 (pEF011), and PikAIV M6 (pKB027) plasmids were transformed into chemically competent Escherichia coli Rosetta Blue (Novagen) cells that contain rare codons that help with expression of proteins. Plasmids were initially grown in 50 mL tubes with 10 mL of Luria Bertani- Miller broth (LB) with 50 μg/mL kanamycin (KAN), 34 μg/mL chloramphenicol (CAM), and 12.5 μg/ mL tetracycline (TET) overnight at 37°C shaking at 250 rpm. A second pass of the cells were grown in 2 liter flasks with 1.5 liters of terrific broth (TB) (Harbor, 2015) with 5% glycerol, 50 μg/mL KAN, 34 μg/mL CAM, and 12.5 μg/ mL TET at 25°C shaking at 200 rpm. Cell densities were measured every hour using a spectrophotometer (Eppendorf, Hamburg, Germany) until cells reached optical density (OD600) of 1. Flasks were then incubated at 16°C for 1 hour to acclimate cells to lower temperature. Rosetta Blue cells were induced with 100 mM IPTG and incubated with shaking at 200 rpm at 16°C for 24 hours.
2.2.2 Extraction of Recombinant KS-AT proteins

Cells were centrifuged at 4,000 rpm at 4 °C for 25 minutes in 750 mL sterile containers and the supernatant was discarded. The protein was extracted from the cell pellet using BugBuster® (Merck Millipore, Darmstadt, Germany) reagent specifications. BugBuster® was diluted 1:10 with QIAexpressionist (Qiagen, Valencia, California, USA) lysis buffer pH 8 consisting of 50 mM sodium phosphate (monobasic), 300 mM sodium chloride, and 10 mM imidazole. Cells were resuspended in the 1X Bugbuster® and incubated with slight agitation for 1 hour at room temperature. The soluble and insoluble protein fractions were separated by centrifugation at 4,000 rpm for 40 minutes. After centrifugation, the supernatant was collected as the soluble protein while the pellet was resuspended in 1X Bugbuster® as the insoluble fraction. All protein fractions were stored at -80 °C.

2.2.3 Purification of Recombinant KS-AT constructs

2.2.3.1 Ni-NTA purification

The target proteins were purified from the total soluble protein fraction with Ni-NTA chromatography. Ni-NTA chromatography was performed by incubating the soluble fraction with Ni-NTA agarose (Qiagen, Hilden, Germany) according to protocol specification of 10 µL resin to 50-100 µg of protein (Qiagen, 2003). The protein - Ni-NTA agarose suspension was incubated at 4°C with slight agitation for 1 hour. Protein samples were transferred to a column where cell lysate was removed by gravity flow leaving only the agarose matrix and attached proteins. The column was washed twice with wash buffer pH 8 consisting of 50 mM sodium phosphate, 300 mM sodium chloride, and 20 mM imidazole. His-tag purified proteins were eluted from the columns using elution buffer pH 8 consisting of 50 mM sodium phosphate, 300 mM sodium chloride, and 250 mM imidazole. Protein fractions were separated using SDS-
PAGE. SDS-Page was performed using 4-20% Mini-PROTEAN® TGX™ (Bio-Rad, Hercules, California, USA) precast gels and a 10-250 kDa protein ladder (NEB, Ipswich, MA, USA) to visualize protein bands.

2.2.3.2 Thrombin Cleavage of 6X His-tag from BryAT₁

BryPAT₁ (pNL020) was concentrated using Amicon® Ultra-4 centrifugal filters (Merck Millipore Ltd., Tullagreen, Cork, Ireland) for 30 minutes at 4,000 rpm at 4°C. pNL020 was exchanged into calcium chloride buffer consisting of 50 mM Tris-HCl, pH 8, and 10 mM calcium chloride by centrifuging at 4,000 rpm for 30 minutes at 4°C in Amicon® Ultra-4 filter. Thrombin cleavage was performed according Thrombin CleanCleave™ Kit (Sigma-Aldrich, St. Louis, Missouri, USA) protocol. His-tagged proteins were suspended in cleavage resin at room temperature with gentle agitation for 4 hours. pNL020 was recovered by supernatant collection using room temperature centrifugation at 2,500 rpm for 5 minutes.

2.2.3.3 Size-Exclusion Chromatography

pNL020, pKB114, and pEF011 were further purified with size-exclusion chromatography (SEC) using a fractionation range of 3000–70000 Da. The column was packed using 15 mL of Superdex® 75 agarose beads (Sigma-Aldrich, St. Louis, Missouri, USA) in a XK16/20 (GE, Uppsala, Sweden) glass column. SEC purification running buffer consisted of 0.2µm filtered 50mM monobasic sodium phosphate, 50mM dibasic sodium phosphate, and 150mM sodium chloride. Purification was performed on an ÄKTA Explorer 100 (GE Healthcare, Malbourgh, MA, USA) at 0.4 mL/min flow rate for approximately 1 hour. All protein fraction volumes
collected were 2 - 4 mL. Each fraction was visualized using SDS-Page to locate the protein size of interest.

### 2.2.3.4 Protein Concentration and Quantification

Protein fractions with the band of interest were concentrated using Amicon® Ultra-4 centrifugal filters for 30 minutes at 4,000 rpm at 4°C. Buffer exchange was performed using HBS-P buffer consisting of 10mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 7.4, 150mM sodium chloride, 0.05% Tween 20, and 0.1% DMSO by centrifugation for 40 minutes at 4,000 rpm at 4°C. Concentrated proteins were then filtered with 0.22 µm low protein binding Durapore® (PVDF) membrane (Merck Millipore Ltd., Tullagreen, Cork, Ireland). Protein concentrations were quantified by Pierce™ bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, Illinois, USA) using bovine serum albumin (BSA) as a standard. The protein concentrations were measured using a Wallac-Victor2 plater reader (Perkin Elmer, Bradford, Connecticut, USA) at 600nm.

### 2.2.4 Protein-Protein Interactions of Recombinant Constructs

#### 2.2.4.1 Surface Plasmon Resonance

The protein-protein interactions were assessed with surface plasmon resonance (SPR) to quantify the binding affinities of BryPAT1 to the different polyketide KS-AT interdomain regions. SPR experiments were performed with BryBM4 and EryAIII M6 KS-AT affixed to Series S Sensor Chip NTA (GE Healthcare) via the 6x-His tag. The NTA sensor chip consists of a gold metal film on one side, and the other side is a dextran matrix to which the 6x-His tag ligand is bound using a nickel chloride solution. The gold metal film is exposed to a polarized light source causing the excitation of electrons on the gold metal surface, resulting in surface...
plasmons. The KS-AT proteins, ligands, are tethered to the chip and were exposed to the analyte, BryP AT₁, as the buffer solution flows across the chip. As the analyte binds to the ligand, the light refraction on the sensor chip changes causing angles of resonance shown as response units (RU). Angles of resonance due to light refraction are measured and analyzed according to association (kₐ), binding of the analyte to the sensor chip ligand, and disassociations (kₐ), and removal of bound analyte from ligand. Data are provided in a sensorgram consisting of plotted data points of RU versus time (seconds). Binding affinity of the analyte and ligand is provided in ability of the analyte to be disassociated (kₐ) from the ligand. The affinity constant Kₐ is calculated by the ratio of rate constants kₐ and kₐ (Kₐ = kₐ / kₐ). The Kₐ was measured at five different analyte concentrations ranging between 0.1 and 10 times the Kₐ of the interaction.

Surface plasmon resonance was performed on a Biacore T-200 (GE Healthcare, Uppsala, Sweden) instrument using a Series S Sensor Chip NTA (Lot # 10250946, GE Healthcare, Uppsala, Sweden). The experimental running buffer consisted of 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 0.15 M sodium chloride (NaCl), and 0.005% Tween 20. Purified proteins derived from pKB114 (41 μg) and pEA011 (54 μg) were bound to the NTA sensor chip via 6X His-tag using a 0.5 mM nickel chloride solution (NTA Reagent Kit, GE Healthcare, Uppsala, Sweden). Proteins were then cross-linked captured onto the NTA chip using 20 μM N-hydroxysuccinimide (NHS) / 5 μM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Amine Coupling Kit, GE Healthcare, Uppsala, Sweden) for 7 min at flow rate of 5 μl/min and excess crosslinkers were quenched by injection of 1 M ethanolamine for 10 min. Any remaining nickel chloride solution was removed using 0.35 M ethylenediaminetetraacetic acid (EDTA) (NTA Reagent Kit, GE Healthcare, Uppsala, Sweden). Single cycle kinetics were measured at 10 hertz (Hz) with a 30 μL/min flow rate for a 60 second
association time and a 600 second dissociation time at 25°C. pNL020 was diluted in HBS-P to achieve experimental analyte concentrations of 0 µM, 0.117 µM, 0.234 µM, 0.467 µM, 0.935 µM, 1.837 µM, 3.75 µM, and 7.5 µM. The NTA chip was regenerated using 0.35 M ethylenediaminetetraacetic acid (EDTA) (NTA Reagent Kit, GE Healthcare, Uppsala, Sweden).

Response units and time were evaluated using Biacore T200 Software v3.1 with parameters of 4 seconds before injection stop and 5 seconds after with a steady state curve fit. Channel one and Channel 3 of NTA chip were used as a reference blanks to subtract background to achieve a baseline for accurate K_D calculations.

2.3 Results

2.3.1.1 KS-AT Construct Sequences and Overexpression Confirmation

KS and AT regions of BryB M4, Ery AIII M6, and PikAIV M6 were located using MotifScan. Regions between the KS and AT regions were recorded and aligned to identify differences in amino acid sequences (Figure 2.1). BryB M5, Ery AIII M5, and Pik AIII M5 modules were added into the alignment to give more strength to the alignment of conserved

![Figure 2.1 Alignment of Bry, Ery, and Pik modules of KS-AT interdomain regions](image)
regions. The differences seen in the amino acid sequence could be used to explain the ability of Bry and Ery modules to accept the BryAT₁. Similarities between Bry and Ery are seen at positions 6, 48, and 55 while Pik differs in these areas. One of these amino acid locations could potentially be the difference in the ability of the AT to load an extender unit onto the Bry and Ery modules and not the Pik.

Previously cloned, BryPAT₁ (pNL020) and Ery AIII M6 (pEA021) constructs were easily transformed and expressed in the Rosetta Blue cells with the protein of interest found in the soluble fraction (Figure 2.1). BryPAT₁ (pNL020) has a protein size of approximately 32.3 kDa while Ery AIII M6 (pEA021) has a size of about 21.2 kDa. The empty pET28b vector was used as a negative control to confirm overexpression in constructs. The PikAIV M6 KS-AT primers

![Figure 2.2: SDS-Page of KS-AT Interdomain Region Constructs](image)

Overexpressed soluble (S) and Ni-NTA (His-tag) purified proteins of EryAIII M6 KS-AT (pEA021, ~21.2kDa), BryBM4 KS-AT (pKB114, ~34.6 kDa), and BryAT₁ (pNL020, ~32.3 kDa) with NEB (10-250 kDa) protein ladder. pET28b lane is the empty vector.
were effective in amplifying the KS-AT interdomain region using PCR. However, the Pik amplicon was unable to be cloned successfully cloned into the pET28b vector. After several attempts, the PikAIV M6 KS-AT plasmid was not able to be successfully transformed into XL1-Blue cells. In an effort to continue with the Pik construct, the PikAIV M6 KS-AT sequence was codon optimized and synthetically constructed by gBlocks® Gene Fragments. The synthetic PikAIV M6 KS-AT sequence was successfully cloned into the pCDF-1b vector. The pCDF-1b:PikAIV M6 KS-AT (pKB027) construct was transformed into both XL1-Blue and Rosetta Blue cell lines, although it failed to produce any protein of interest in the soluble fraction after multiple attempts. BryBM4 KS-AT (pKB114) (Figure 2.2) was cloned into the pET28b vector and expressed in the Rosetta Blue cells. pKB114 has a size of about 34.6 kDa which is slightly larger than pEA021 construct due to the primer combination design. pKB114 consists of a small region of KS domain as well as the KS-AT interdomain in the construct whereas pEF011 consists of just the KS-AT interdomain region.

2.3.1.2 KS-AT Construct Purified Proteins and Concentrations

Proteins overexpressed from BryPAT1 (pNL020), EryAIII M6 KS-AT (pEA021), and BryB M4 KS-AT (pKB114) are present at the approximate size markers with little to no contaminating bands in the samples. Protein concentrations of the purified constructs were adequate for the numerous SPR experiments. Each SPR experiment required approximately 30 ng of protein. From 2 liters of culture, pNL020 had a yield of 435.17 µg/mL of protein. pEA021 had 365.4 µg/mL, while pKB114 had the best yield with 548.8 µg/mL of protein.

2.3.1.3 SPR Experiments of pNL020 with pKB114 and pEA021

Initially, SPR experiments were unsuccessful due to unknown non-specific binding on NTA chip surface. Once protein constructs were cross-linked immobilized onto the NTA chip
experimental data was more valid. Surface density of protein attachment to the NTA chip for pKB114 was 236.3 RU while pEA021 was 153.6 RU. Data for the $K_D$ values obtained during the SPR experiments with pNL020 as the analyte were plotted response (response units) versus concentration (M) (Figure 2.3 & 2.4). $K_D$ raw data of binding affinities for each experiment was recorded (Table 2.2). BryAT1 (pNL020) binding affinities to EryAIII M6 (pEA021) were normalized to the $K_D$ values of BryB M4 (pKB114) interaction with BryAT1 (pNL020) (Table 2.3). Normalized values ranged from 0.68 to 23.98 with an average of 7.48. Standard deviation of these values was 9.33.

Figure 2.3: SPR Experiment of BryBM4 KS-AT (pKB114) versus BryPAT1 (pNL020)

![Graph showing SPR experiment results](image)

pKB11-4 exposed to 0 µM, 0.117 µM, 0.234 µM, 0.4675 µM, 0.935 µM, 1.84 µM, 3.75 µM, 7.5 µM, and 15 µM of pNL020 plotted with response units (RU) versus concentration in molarity (M). KD value of 2.30E-7.
**Table 2.2: $K_D$ Values of SPR cycle data from BryAT1 versus pKB114 and pEA021**

<table>
<thead>
<tr>
<th>KS-AT Construct</th>
<th>$K_D$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKB114</td>
<td>5.23E-08</td>
</tr>
<tr>
<td></td>
<td>8.25E-09</td>
</tr>
<tr>
<td></td>
<td>2.30E-07</td>
</tr>
<tr>
<td></td>
<td>6.97E-09</td>
</tr>
<tr>
<td></td>
<td>2.44E-08</td>
</tr>
<tr>
<td>pEA021</td>
<td>3.55E-08</td>
</tr>
<tr>
<td></td>
<td>3.02E-09</td>
</tr>
<tr>
<td></td>
<td>1.34E-07</td>
</tr>
<tr>
<td></td>
<td>8.22E-08</td>
</tr>
<tr>
<td></td>
<td>5.84E-07</td>
</tr>
</tbody>
</table>

Figure 2.4: SPR Experiment of Ery AIII M6 KS-AT (pEA021) versus BryPAT1 (pNL020)

pEA021 exposed to 0 µM, 0.117 µM, 0.234 µM, 0.4675 µM, 0.935 µM, 1.84 µM, 3.75 µM, 7.5 µM, and 15 µM of pNL020 plotted with response units (RU) versus concentration in molarity (M). $K_D$ value of 8.221E-8.
Table 2.3: SPR data normalized to pKB114

<table>
<thead>
<tr>
<th>pEA021 K(_D) normalized to pKB114</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>11.79</td>
</tr>
<tr>
<td></td>
<td>23.98</td>
</tr>
<tr>
<td><strong>Average K(_D)</strong></td>
<td>7.48</td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td>9.33</td>
</tr>
</tbody>
</table>

2.4 Discussion

\(K_D\) values are unitless numbers described as if they have molar units of concentration.

Non-covalent interactions using ranged from picomolar to nanomolar for the strongest interactions while millimolar is found with weaker interactions. Signaling proteins binding to molecules usually have \(K_D\) values in the micromolar range (Kuriyan, 2008). Binding events in the SPR experiments show that BryAT\(_1\) (pNL020) does interact in the interdomain region between KS and AT in EryAIII M6 (pEA021) and BryB M4 (pKB114). \(K_D\) values range from \(10^{-7}\) to \(10^{-9}\) which fall into the micromolar to nanomolar range and could indicate successful binding of the BryAT\(_1\) to the KS-AT interdomain regions. Previous research has shown that BryAT\(_1\) has the ability to load a malonyl-CoA onto module BryBM4 and EryAIII M6 (Lopanik
but the mechanism for doing so has not been elicited. The potential binding events in the KS-AT interdomain region could be region of the module in which the BryAT₁ interacts to load the malonyl-CoA to the growing nascent polyketide chain. Specific amino acid residues that are involved in the potential binding events are unknown but alignments of the KS-AT interdomain regions of BryBM4, EryAIII M6, and Pik AIV M6 (Figure 2.1) show different amino acids that could be used for recognition by the trans-AT. BryBM4 and EryAIII M6 have a leucine in position 6 and 48 while Pik AIV M6 has a valine and an alanine, respectively. Also, amino acid residue 55 is a serine for Pik AIV M6 while BryB M4 and Ery AIII M6 have glycine. While the leucine, alanine, and valine are all hydrophobic amino acids and usually found on the core backbone of the protein, they do differ in size slightly which could cause conformational changes in the protein. The difference of a glycine to a serine could be more impactful due to the change from a hydrophobic amino acid to a polar amino acid that can participate in hydrogen bonding. The Pik AIV M6’s serine at residue 55 could have properties that would create conditions where the BryAT₁ could not interact with the module.

2.5 Conclusion

K₅ values show differences in BryAT₁’s ability to interact with BryB M4 and Ery AIII M6 with normalized value of 7.48. The data also indicates that protein-protein interactions could be occurring in this region of the PKS module. Successful cloning and overexpression of the Pik AIV M6 KS-AT region would help to further provide evidence for this hypothesis. Binding affinity data of Pik AIV M6 KS-AT region exposed to BryAT₁ could show if interactions are possible and if so, potential amino acids that might be involved. Site-direct mutagenesis of the amino acid residues of interest would also help to demonstrate which amino acids appear to be involved in the protein-protein interactions. SPR experiments would be repeated once the
correct substitutions have been made in these areas to determine changes in the binding affinities. Changes in SPR data from amino acid substitutions would supplement this idea.
3 IDENTIFY AND COMPARE PROTEIN KINASE C C1B DOMAINS FOUND IN

BUGULA SPP.

3.1 Introduction

Natural products produced by microorganisms can be potent prokaryotic and eukaryotic cell effectors. For instance, the anticancer compound epothilone produced by the myxobacterium Sorangium cellulosum inhibits microtubule assembly, and salinosporamide, isolated from the marine bacteria Salinispora spp. inhibits proteasome function (Cragg & Newman, 2013). Thus, the majority (75%) of approved therapeutic agents are either natural products or their derivatives (Newman & Cragg, 2016), most of which are produced by microorganisms. In many cases, natural products obtained from a metazoan are actually produced by a microbial symbiont (reviewed in (Piel, 2009)). One example of this is the compound rhizoxin, that is associated with the fungus that produces rice blight. This compound was shown to be produced by a endosymbiotic bacterium that associates with the fungus (Partida Martinez & Hertweck, 2005). Rhizoxin was investigated as an anticancer agent because of its ability to bind tubulin (Hendriks et al., 1992). As the fungal host possesses tubulin, the question of how the host is able to grow in the presence of bacterially-produced rhizoxin arose. Interestingly, the fungal tubulin protein was found to possess a critical amino acid substitution which decreased the binding affinity of rhizoxin (Schmitt et al., 2008). Further, this amino acid substitution was absent in related fungal strains that did not host the symbiotic bacterium.

Bugula neritina is the source of the bryostatins, macrocyclic polyketides that modulate protein kinase C activity. The bryostatins bind with high affinity to the regulatory C1b region of PKC (Wender et al., 1988, Wender et al., 2011). The bryostatins have been shown to be
produced by an uncultured symbiont, “Cand. E. sertula.” *B. neritina* is a complex of multiple closely related sibling species (Davidson and Haygood 1999, McGovern and Hellberg 2001), and interestingly, some individual colonies have been shown to not possess the symbiont (Linneman et al. 2014). Investigations into other species of *Bugula* have demonstrated that some possess a related symbiont that produces bryostatin-like molecules (*B. simplex*, Lim and Haygood 2004), whereas others possess a related symbiont that does not produce bryostatin-like molecules (*B. pacifica, B. turbinata*, Lim-Fong et al. 2008), and some do not possess a symbiont or bryostatins (*B. stolonifera*, some colonies of *B. neritina*, Woollacott 1981, Linneman et al. 2014). This variation in symbiont/bryostatin presence among closely related *Bugula* species provides an opportunity to investigate the impact of host-symbiont coevolution in the presence of the symbiont-produced natural products. As bryostatins modulate PKC activity, it is possible that PKCs in hosts with symbiont-produced bryostatins respond differently to their presence as a result of coevolution, and the PKC-affected pathways could influence the *Bugula* spp. systems and physiology. I hypothesize that PKCs in *Bugula neritina* and other *Bugula* spp. that have symbiont-produced bryostatins have evolved under different selection pressures than PKCs in *Bugula* spp. without bryostatins.

To investigate this possibility, PKC C1b domains were sequenced and compared in *B. neritina* (aposymbiotic and symbiotic) colonies as well as other *Bugula* spp. that do and do not possess the symbiont and/or the bryostatins to determine if PKCs from *Bugula* species that evolved in the presence of bryostatins have modified bryostatin-binding domains. It is possible that variations in the C1b domain may result in differing binding capabilities between PKC isoforms and bryostatins within *Bugula* species. In order to analyze these interactions, PKC C1b domains were compared among colonies of differing *Bugula* spp. Because very little sequence
data for *B. neritina* exists, a transcriptomics approach was used to assess the diversity of PKCs. PKC C1b genes were confirmed via PCR amplification using primers designed according to the transcriptomic data and sequenced. This approach was also used to obtain partial sequences of PKCs from the other species of *Bugula*. Sequences were analyzed and amino acid alignments evaluated for conserved amino acids between the species and symbiotic types. I hypothesize that PKC C1b domain regions will vary in amino acid residues between each *Bugula* species. Differences in C1b domain sequences in host species would support the hypothesis that the host PKCs have evolved under different conditions such as the presence of the symbiont-produced bryostatin compounds.

### 3.2 Materials and Methods

#### 3.2.1 Sample Collection and Genotyping of *Bugula neritina*

*Bugula* spp. were collected from the Morehead City, NC, preserved in RNA Later, and frozen at -20°C. The collected colonies were identified to species using the host mitochondrial cytochrome c oxidase I sequence (Davidson & Haygood, 1999, McGovern & Hellberg, 2003). Genomic DNA was extracted using ZR Fungal/Bacterial DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA) and quantified using a Nanodrop™ ND-1000 spectrophotometer (Nanodrop™ Technologies, Inc., Wilmington, Delaware, USA). DNA was PCR amplified using Phire Hot Start II DNA polymerase (Thermo Scientific, Pittsburgh, Pennsylvania, USA) and BnCOI F and BnCOI R primers (Table 3.1) (Linneman *et al.*, 2014). PCR reactions were carried out by initial denature at 98°C for 30s sec, with 35 cycles of denatured at 98°C for 5 sec, annealed for 30 sec at 59°C, and elongated at 72°C for 25 sec, and final extension of 72°C for 2 min. PCR amplicons were digested for 30 minutes at 37°C with AseI and AluI (NEB, Ipswich,
MA, USA) and then deactivated at 80°C for 20 minutes. Digested products were separated using agarose gel electrophoresis and analyzed for band sizes specific to either Type S or Type N B. neritina.

3.2.2 Transcriptomics of Bugula neritina

Transcriptomic data was performed previously (Mathew et al., 2016) by symbiotic and naturally symbiont-reduced Type S B. neritina zooids preserved in TRIzol reagent (Invitrogen, Carlsbad, California, USA). Total RNA was extracted as per the manufacturer’s instructions and purified using RNeasy Mini kit (Qiagen, Valencia, California, USA), and RNase-free DNase I treated to remove contaminating genomic DNA. RNA quality was determined using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, California, USA) and found to have a RNA integrity number (RIN) value of 8. The purified total RNA was processed according to TruSeq RNA Sample Preparation Kit (Illumina, San Diego, California, USA) procedure for preparation of mRNA library. The adapter-ligated cDNA library was hybridized to Illumina

Table 3.1 Primers used in Genotyping of Bugula spp.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Target</th>
<th>Product size (bp)</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>BnCOI F</td>
<td>ACAGCTCATGCACTTTTTTA</td>
<td>B. neritina COI gene</td>
<td>469</td>
<td>59°C</td>
</tr>
<tr>
<td>BnCOI R</td>
<td>CATTACGATCGGTTAGTAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCO 1490 F</td>
<td>GGTCACACAAATCATAAAGATATTGG</td>
<td>Mitochondrial (COI) genes</td>
<td>710</td>
<td>50°C</td>
</tr>
<tr>
<td>HCO 2198 R</td>
<td>TAAACTTCAGGGTGACAAAAAATCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT 16SAR F</td>
<td>CGCCTGTTTATCAAAAAACAT</td>
<td>Mitochondrial 16S gene</td>
<td>552</td>
<td>50°C</td>
</tr>
<tr>
<td>MT 16SCB R</td>
<td>CCGGTCTGAACTCAGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
flow cell and sequenced on an Illumina HiSeq 2500 sequencing platform (Illumina, San Diego, California, USA) at the Integrated Genomics Facility (Georgia Regents University Cancer Center, Augusta, Georgia, USA). The paired-end reads were assembled De Novo using Trinity software package (version r2013-02-25)(Grabherr et al., 2011), and the assembled contigs were annotated by performing BLASTx searches (Translated Query-Protein Subject BLAST 2.2.26+) against the Swiss-Prot database. Sequences identified as PKCs were further analyzed by MotifScan (ExPASy) to identify relevant domains (Mathew et al., 2016). Based on the sequences identified as PKCs, B. neritina-specific PKC primers were designed (Table 3.2).

3.2.3 Bugula spp. RNA extraction and cDNA synthesis

Once colonies were genetically-typed, 50 mg of each Bugula colony was washed with washed in PBS buffer (Harbor, 2006). The sample was split into two 25 mg aliquots and transferred into separate tubes of 500 µL of TRIzol reagent for homogenization. After ten rounds of homogenization on ice using a sterile pestle, split samples were recombined and RNA extracted. Total RNA was extracted and purified per TRIzol manufacturer’s protocol. After RNA extraction, samples were purified of any PCR inhibitors by using a resin in OneStep PCR inhibitor removal kit (Zymo Research Corp., Irvine, CA, USA). Samples were subjected to an additional DNase I treatment for 1 hour at 37°C (Roche, Mannheim, Germany) and reaction was quenched with the addition of 0.5 M EDTA. Samples were further purified using RNA Clean & Concentrator™ (Zymo Research Corp., Irvine, California, USA). The Nanodrop spectrophotometer was used to quantify RNA concentrations and samples were stored at -80°C. Single stranded cDNA was generated from approximately 2 µg of total RNA using random hexamer primers and the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad,
CA, USA). No reverse transcriptase enzyme controls were performed to ensure no genomic DNA contamination. Confirmation of synthesized cDNA was performed using PCR with mitochondrial 16S gene primers (Braga et al., 1999) (Table 3.1) and Phire Hot Start II polymerase. PCR reactions were carried out by initial denature at 98°C for 30s sec, with 35 cycles of denatured at 98°C for 5 sec, annealed for 30 sec at 50°C, and elongated at 72°C for 25 sec, and final extension of 72°C for 2 min.

3.2.4 Amplification of PKC genes in Bugula neritina and Bugula spp.

Using transcriptomics data, primers specific to PKC isomers were designed to flank C1b domain regions according to B. neritina Type S C1b domain data using the NCBI Primer-BLAST (Ye et al., 2012) tool (Table 3.3). The α, ε, and δ isomers were targeted for amplifying the C1b domain region while ι isomer was amplified for the absence of the C1b domain. cDNA using Phire Hot Start II DNA polymerase and B. neritina-specific PKC primers (Mathew et al., 2016). PCR reactions were carried out by initial denature at 98°C for 30s sec, with 35 cycles of denatured at 98°C for 5 sec, annealed for 30 sec (temperature varied by primer set), and elongated at 72°C for 25 sec, and final extension of 72°C for 2 min. PCR amplicons were analyzed by agarose gel electrophoresis, and reactions resulting in single amplicons were column purified (GeneJET PCR Purification kit). PCR reactions yielding multiple PCR amplicons will be purified using gel purification (GeneJET Gel Extraction kit) and were sequenced at the DNA Analysis Facility at Yale University, New Haven, CT, USA.

3.2.5 Sequence Analysis

Sequences of the amplified PCR products were edited for consensus sequences. The C1b domain sequences were identified by domain analysis and aligned with other known C1b domain sequences from multiple species using ClustalX v2.0 (Larkin et al., 2007). Genetic
relationships of the PKC C1b domain isomers were assessed via phylogenetic analysis using

MEGA (Tamura et al., 2013).

Table 3.2 Primers used in identification of PKC isoforms in Bugula spp.

<table>
<thead>
<tr>
<th>Combinations of PKC C1b domain primers</th>
<th>DNA Size (bp)</th>
<th>AA length</th>
<th>Target</th>
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</thead>
<tbody>
<tr>
<td>BnPKCd_1f</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>976</td>
<td>325</td>
</tr>
<tr>
<td>BnPKCd_1000r</td>
<td>TACAGACGCTGCAATGATCTGCATAC</td>
<td>1026</td>
<td>311</td>
</tr>
<tr>
<td>BnPKCe_1f</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>1084</td>
<td>315</td>
</tr>
<tr>
<td>BnPKCe_1027r</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>622</td>
<td>207</td>
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<tr>
<td>BnPKCe_484f</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>543</td>
<td>154</td>
</tr>
<tr>
<td>BnPKCe_466f</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>561</td>
<td>164</td>
</tr>
<tr>
<td>BnPKCa8617_10f</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>1000</td>
<td>329</td>
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<tr>
<td>BnPKCa8617_1000r</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>541</td>
<td>180</td>
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<tr>
<td>BnPKCa8617_237f</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>833</td>
<td>273</td>
</tr>
<tr>
<td>BnPKCa8617_6484_174f</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>546</td>
<td>182</td>
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<tr>
<td>BnPKCa8617_6484_237f</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>432</td>
<td>144</td>
</tr>
<tr>
<td>BnPKCa8617_6484_668r</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>901</td>
<td>301</td>
</tr>
<tr>
<td>BnPKCa8617_6484_77f</td>
<td>ATGTCGGTGGTTGGTTTTATCC</td>
<td>692</td>
<td>231</td>
</tr>
</tbody>
</table>

Table 3.2 Primers used in identification of PKC isoforms in Bugula spp.
3.3 Results

3.3.1 Confirmation of sequences

Transcriptomics analysis of Type S *B. neritina* sequences yielded at least 5 expressed PKC isoforms. Bioinformatic analysis of the PKC contigs indicated that contigs 4634 and 16020 were homologous to conventional αPKCs. The two novel PKCs found were contig 12712, a δ isoform, and contig 16336, an ε isoform. The analysis also specified one atypical ι-type PKC, contig 6484 (Mathew et al., 2016). RT-PCR analysis of symbiotic *B. neritina* Type S confirmed the presence of these PKC isoforms (Mathew et al., 2016). The isomers of conventional αPKCs, novel PKCs (δ and ε), and atypical ι-type PKC were successfully amplified in *B. neritina* type S and sequenced. Sequences analyzed via highest hit in BLASTp (Altschul et al., 1997) confirmed that contigs 4634 was a 59% identity match to a PKC α isoform found in *Macaca mulatta*, while contig 16020 matched with 61% identity to PKC α type-like isoform X1 in *Haplochromis burtoni*. The highest BLASTp hit for contig 12712 shared a 55% sequence match with a protein kinase C δ type-like characterized in *Saccoglossus kowalevskii*. Contig 16336’s highest hit was 63% identical to a calcium-independent protein kinase C in *Aplysia californica*. The atypical ι PKC isomer contig 6484 was found to have a 72% identity match to its highest hit of *Lymnaea stagnalis* (Table 3.4). Confirmed *Bugula neritina* Type S PKC sequences were submitted to National Center for Biotechnology Information (NCBI) Genbank® database (Benson et al., 2013). Contigs of all 5 *B. neritina* PKC cDNA sequences have been issued accession numbers KM047237 to KM047241.
### Table 3.3 PKC homologs identified by Bugula neritina transcriptome sequencing

<table>
<thead>
<tr>
<th>Contig</th>
<th>DNA length</th>
<th>Amino acid length</th>
<th>Highest hit (by blastp)</th>
<th>e-value</th>
<th>% identity</th>
<th>PKC isoform</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4634</td>
<td>2130</td>
<td>710</td>
<td>Protein kinase C α type (Macaca mulatta)</td>
<td>0.0</td>
<td>59%</td>
<td>Conventional α</td>
<td>NP_001247662</td>
</tr>
<tr>
<td>16020</td>
<td>2031</td>
<td>677</td>
<td>Protein kinase C α type-like isoform X1 (Haplochromis burtoni)</td>
<td>0.0</td>
<td>61%</td>
<td>Conventional α</td>
<td>XP_005942808</td>
</tr>
<tr>
<td>12712</td>
<td>2091</td>
<td>697</td>
<td>Protein kinase C δ type-like (Saccoglossus kowalevskii)</td>
<td>0.0</td>
<td>55%</td>
<td>Novel δ</td>
<td>XP_002740313</td>
</tr>
<tr>
<td>16336</td>
<td>2148</td>
<td>716</td>
<td>Calcium-independent protein kinase C (Aplysia californica)</td>
<td>0.0</td>
<td>63%</td>
<td>Novel ε</td>
<td>NP_001191401</td>
</tr>
<tr>
<td>6484</td>
<td>1710</td>
<td>570</td>
<td>Atypical protein kinase C (Lymnaea stagnalis)</td>
<td>0.0</td>
<td>72%</td>
<td>Atypical ι</td>
<td>BAK09601</td>
</tr>
</tbody>
</table>

### 3.3.2 Analysis of C1b domains in B. neritina PKC isoforms

Sequences of PKC C1b domains, 50 amino acids in length, from the Type S B. neritina were compared for differences in the amino acid residues that could be important for PKC activation. The C1b domains of the highest BLAST hits and nearest neighbors of the 5 contigs were compared to those from B. neritina. Using MegAlign, the amino acid sequences of C1b domains were aligned and compared to identify conserved residues and difference between the PKC isoforms of B. neritina and BlastX highest hits (Figure 3.1). All contigs of conventional,
novel and atypical PKCs had several regions of highly conserved regions. Residues 3 in the
novel ε PKC C1b domains exhibit several variations. PKC α isforms show variability at residues
5 and 43. PKC δ isoforms have dissimilarities at position 45 but the most obvious difference is at
position 12. *B. neritina* differs with a histidine at position 12 unlike the rest of the PKC δ C1b
domains shown. Also, while the PKC δ is also a novel PKC, it has a higher homology with the
conventional α PKC than that of the ε. Phylogenetic analysis (Figure 3.2) depicts the genetic
relationships of the confirmed *B. neritina* PKC c1b domains in comparison with their Blastx
highest hits. The constructed tree shows that PKC conventional α and novel δ are the more
closely related while the novel ε has less homology to δ. PKC ι isoforms are the most distantly
related.

*Figure 3.1: Alignment of C1b domains from B. neritina PKC isoform contigs and highest BlastX hits*
Figure 3.2: Phylogenetic tree of B. neritina PKC isoform contigs and highest BlastX hits
3.4 Discussion

From the transcriptome sequences, five contigs from differing isoforms of PKCs were identified in *B. neritina* cDNA sequences. These genes have a high degree of homology with a diverse group of organisms including *Macaca mulatta* primate, *Haplochromis burtoni* fish, *Saccoglossus kowalevskii* hemichordates, and molluscs spp. *Aplysia californica* and *Lymnaea stagnalis* (Table 3.3). Several residues such as His1, Leu21, Gly23, Cys50, positions 14-18 are conserved amongst all of the sequences from *B. neritina* and the other organisms. In residues 4 and 10, novel PKC δ C1b domain to have a higher homology with conventional PKC α than the novel ε isoform PKCs (Figure 3.1). Phylogenetic analysis (Figure 3.2) confirms that PKC δ of *B. neritina* contig 12712 is more closely related to the PKC α conventional contigs of 4634 and 16020; however, there was not strong bootstrapping to support. While the atypical PKC ι isoforms were confirmed in *B. neritina* (Table 3.2), this group only contains a C1a domain in the DAG binding region and lacks the C1b domain where bryostatin-1 binds to activate PKC phosphorylation. PKCs δ and ε are more similar at amino acid position 7 and 26 than the α isoform.

The histidine amino acid residue at position 12 of *B. neritina* contig 12712 and *C. teleta* differs with a serine, the deviation at this position while all other PKCs have a threonine. Histidine, serine, and threonine are all polar amino acids; however, the histidine side chain is positively charged while serine and threonine are uncharged. Also, mouse nPKC δ had potential to participate in hydrogen binding at carbonyl of Met9, the amino of Thr12, the carbonyl of Leu21, and the amino of Gly23 (Kimura *et al.*, 1999). PKC δ C1b domain of *B. neritina* shows the last Leu21 and Gly23 are conserved; however, Met9 is an Ile and Thr12 is a His (Figure 3.1). The difference in the amino acids could be due to evolutionary change in *B. neritina* due to the
presence of bryostatin compounds produced by the symbiont. Experimental analysis of the bryostatins ability to bind the \textit{B. neritina} C1b domains, with amino acid exchanges, could highlight importance of specific residues for PKC activation.

3.5 Conclusion

Amino acid residues involved in the activation of PKC’s in \textit{B. neritina} C1b domain sequences are unknown. Analysis of the C1b domains could discover which amino acids involved in binding of the natural product bryostatin to the DAG binding region. SPR binding affinity experiments of \textit{B. neritina} C1b domains and other organisms could detect specific residues involved in this recognition. Sequence data of PKC δ has identified Thr12 as well conserved among the conventional C1b domains; however, \textit{B. neritina} nPKC δ and the annelid, \textit{Capitella teleta} differ at that position with a histidine and serine, respectively. In addition, variations in the Met9 residue of the ePKC α domain sequences could also provide areas for binding analysis to bryostatins. Experimental analysis of bryostatin binding to C1b domains with modified amino acid residues will allow us to understand the importance of these residues for PKC activation. Previous research has suggested that bryostatin 1 binds to the peptide backbone of the protein and not functional groups.

Further work should be conducted to identify the PKC C1b domains in other \textit{Bugula} spp. to detect differences in sequences. Possible differences could be due to an evolutionary change due to the presence of bryostatins. Other \textit{Bugula} spp. such as \textit{B. pacifica} do not contain a bryostatin-producing symbiont. To characterize the effects bryostatin natural products have on the PKC C1b domains of the host \textit{Bugula neritina}, with bryostatins, and other \textit{Bugula} spp., without bryostatins, to determine if these amino acids could be involved in bryostatin activation.
Amino acid residues of 9Ile and 12His could be areas for amino acid substitution to provide further evidence of activation due to binding at the positions.
4 QUANTIFY BRYOSTATIN AFFINITY FOR *BUGULA* SPP. PKC C1B DOMAINS

4.1 Introduction

*Bugula* spp. vary in symbiont and bryostatin status with some species possessing a symbiont that produces bryostatins, some species possessing a closely-related symbiont that does not produce bryostatins, and some species with no symbiont or bryostatins. The presence of bryostatin could directly affect the *Bugula* host. PKC C1b domain gene evolution could have occurred due to the presences of the natural products. Through the identification of PKC C1b domains in Type S *B. neritina* and *Bugula* spp. possible amino acids changes can be identified. The identification of amino acid variations between species with bryostatins and without could to understanding of bryostatin affinity for the C1b domains.

Sequence differences in the *Bugula* spp. C1b domain regions could result in different binding affinities that are adaptations to the presence of symbiont-produced bryostatins. To investigate this, C1b domain regions of PKC isoforms were amplified from *Bugula* spp. and sequenced. C1b domains from differing PKC isoforms were cloned into a vector for protein expression and the resulting proteins were purified. Their binding affinity for bryostatin and PMA, a similar PKC activator, were quantified. Previous research found that PMA activates PKCs by predominately phosphorylating serine residues (Roush *et al.*, 1999). Surface plasmon resonance was utilized to assess binding differences by quantifying the bryostatin interactions among the *Bugula* spp. C1b domains. I hypothesize that binding affinities in aposymbiotic colonies will be higher than symbiotic colonies. Prolonged exposure to bryostatins in symbiotic *Bugula* species could elicit lower affinities so that the PKC can still be activated. Lower binding
affinities in symbiotic than aposymbiotic Bugula spp. will confirm that PKCs in Bugula spp. with symbiont-produced bryostatins have co-evolved and adapted to the presence of bryostatin. Using alignments of the C1b domains of the Bugula spp. (Figure 3.2), site directed mutagenesis was performed on amino acid residues of PKC C1b domains that vary in sequence. Bugula spp. C1b domains with high and low levels of bryostatin binding and that differ in amino acid residues will be mutated into amino acids more similar to PKC C1b domains of opposite binding affinities. I hypothesize that changes in amino acids could alter bryostatin binding affinity to the PKC C1b domain. Subsequent SPR experiments will allow for data on the changes in bryostatin binding affinities in overexpressed C1b domain proteins of Bugula spp. symbiont-produced bryostatins, symbionts with no bryostatins, and aposymbiotic colonies. Identification of key amino acids for PKC C1b bryostatin binding could lead to understanding host-symbiont interactions and the ability of that interaction to, over time, result in co-evolution to increase host fitness.

4.2 Materials and Methods

4.2.1 Identification of PKC C1b domain in Bugula spp.

4.2.1.1 Sample collection and Genotyping

Bugula spp. of interest were collected from the Pacific Northeast coastlines at Skyline marine center, WA, Deception Pass, WA, Newport marina, WA, Charleston, OR, and Coos Bay, OR. Bugula spp. were also collected from Atlantic Northwest coastlines in Woods Hole (Eel pond), MA, Gloucester, MA, and Portland, MA. All collected samples were preserved in RNA Later and frozen at -20°C. Genomic DNA was extracted using ZR Fungal/Bacterial DNA
MiniPrep™ kit (Zymo Research Corp., Irvine, CA, USA) and PCR amplified as previously described using mitochondrial cytochrome c oxidase, primers LCO1490 and HCO 2198 (Folmer et al., 1994) using Phire Hot Start II polymerase. PCR amplicons were purified using GeneJET PCR purification and were visualized using gel electrophoresis. The PCR amplicons were sequenced at the DNA Analysis Facility at Yale University, New Haven, CT, USA. Consensus sequences were analyzed using BLAST (Altschul et al., 1990) to confirm sample identity.

### 4.2.1.2 Amplification of PKC C1b domains in Bugula spp.

Genotyped Bugula spp. colonies were washed with PBS buffer (Harbor, 2006) then homogenized in TRIzol reagent. Total RNA was extracted per TRIzol manufacturer’s protocol. Samples were purified using OneStep PCR inhibitor removal kit and a DNase I treatment for 1 hour at 37°C (Roche). Samples were further purified using RNA Clean & Concentrator™ and quantified using Nanodrop spectrophotometer. Single stranded cDNA was synthesized using approximately 2 µg of total RNA with SuperScript III First-Strand Synthesis System and random hexamer primers. No reverse transcriptase enzyme controls were performed to ensure cDNA was free of genomic DNA contamination. Confirmation of synthesized cDNA was performed using PCR with mitochondrial 16S gene primers (Braga et al., 1999) (Table 3.1) and Phire Hot Start II polymerase.
Table 4.1: PKC C1b domain primers used to amplify in Bugula spp.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Target</th>
<th>DNA Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BnPKCd_12712_512f</td>
<td>GACAGCCAGTGTCTGTCTTTCT</td>
<td>PKC δ C1b domain (703-852 bp)</td>
<td>541</td>
</tr>
<tr>
<td>BnPKCd_12712_1052r</td>
<td>TGTGCATCTCTGTCTGTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BnPKCd_12712_553f</td>
<td>GGGCTGAACAAGCAAGGAT</td>
<td></td>
<td>433</td>
</tr>
<tr>
<td>BnPKCd_12712_985r</td>
<td>CATCATCCTCCTTTCATTTGCT</td>
<td>PKC δ C1b domain (706-855 bp)</td>
<td>529</td>
</tr>
<tr>
<td>BnPKCe_16336_517f</td>
<td>CAACCAACATTCTGCTTCCCA</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BnPKCe_16336_571f</td>
<td>CAAGGTTACCAATGTCAAGTGTG</td>
<td>PKC ε C1b domain (280-429 bp)</td>
<td>418</td>
</tr>
<tr>
<td>BnPKCe_16336_988r</td>
<td>TATTTACCGAGAGTTGTGGGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BnPKCa_4634_128f</td>
<td>ACCACAAGTTGTACCAGG</td>
<td>PKC α C1b domain (325-474 bp)</td>
<td>534</td>
</tr>
<tr>
<td>BnPKCa_4634_670r</td>
<td>GACTGTGTCGCGACTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BnPKCa_4634_178f</td>
<td>TGCAAGATTTTATTGTGCGG</td>
<td>PKC α C1b domain (280-429 bp)</td>
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<tr>
<td>BnPKCa_4634_611r</td>
<td>TTTACGTAGGGGTCAGCCAG</td>
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</tr>
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<td>BnPKCa_16020_150f</td>
<td>GGGAGTAATTTGGGAAAACAAGGT</td>
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<tr>
<td>BnPKCa_16020_566r</td>
<td>TTGACATACGGATCCTGCAAGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primers used in C1b domain amplification were designed according to the sequences of PKC C1b isoforms from *B. neritina*. Phire Hot Start II polymerase was used in amplifying reactions of an initial 98°C for 30s sec, with 35 cycles of denatured at 98°C for 5 sec, annealed for 30 sec (temperature varied by primer set), and elongated at 72°C for 25 sec, and final extension of 72°C for 2 min. PCR amplicons were analyzed by agarose gel electrophoresis, and reactions resulting in single amplicons were column purified. PCR reactions yielding multiple PCR amplicons were purified using gel purification. Purified PCR amplicons were sequenced at
the DNA Analysis Facility at Yale University, New Haven, CT, USA. *Bugula* spp. PKC C1b domain sequences were confirmed by BLAST and sequence alignments with other C1b domains.

### 4.2.1.3 Construction of PKC C1b domain Plasmids

PKC C1b domain amplicons were digested with *Eco*RI and *Xho*I (NEB, Ipswich, MA, USA) for 2 hours at 37°C and visualized using gel electrophoresis to ensure proper DNA size. The digested DNA band was excised and purified using GeneJET gel extraction kit (Thermo Scientific, Vilnius, Lithuania). Purified PCR-amplified C1b domains were ligated into *Eco*RI and *Xho*I digested a pET28B vector (Novagen). The ligated products were transformed into chemically competent *E. coli* XL1-Blue (Stratagene) cells, plated on LB agar with 50 µg/mL KAN, and incubated at 37°C overnight. Resulting colonies were screened for insert by colony PCR using T7 and T7 terminator primers and One Taq Polymerase (NEB). Colony PCR amplicons of PKC C1b domain isoforms with the size of interest were extracted using *Zyppy™* Plasmid Miniprep Kit (Zymo Research Corp., Irvine, CA, USA) and quantified using Nandrop. Constructs had 6x His-tags located at the N-terminus and the C-terminus of each protein.

### 4.2.2 Construction of PKC δ C1b Domain Mutants

#### 4.2.2.1 Site-direct Mutagenesis

Site-directed mutagenesis was used to alter specific amino acid residues of the PKC δ C1b constructs using primers designed for the position changes. An exponential amplification reaction using Q5 Hot Start High-Fidelity 2X Master Mix (NEB, Ipswich, MA, USA) and approximately 25 ng/µl of construct was performed with initial denaturation at 98°C for 30 seconds, then 25 Cycles at 98°C for 10 seconds, annealing temperature specific to each primer set for 30 seconds, extension at 72°C 20 seconds with a final extension at 72°C for 2 minutes.
Table 4.2: PKC δ C1b Domain Primers for Mutant Constructs

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat_9MI_f</td>
<td>ATAACTACATCAGCCCCACCTTC</td>
<td><em>R. norvegicus</em> C1b domain</td>
</tr>
<tr>
<td>Rat_9MI_r</td>
<td>AGACCTTGAATCGGTGAG</td>
<td>AA residue 9 M→I</td>
</tr>
<tr>
<td>Rat_9ML_f</td>
<td>CTATAACTACCTGAGCCCCACC</td>
<td><em>R. norvegicus</em> C1b domain</td>
</tr>
<tr>
<td>Rat_9ML_r</td>
<td>ACCTTGAAATCGGTGAGGC</td>
<td>AA residue 9 M→L</td>
</tr>
<tr>
<td>Bp_9LM_f</td>
<td>GCACAACATCAGCAACACATTC</td>
<td><em>B. pacifica</em> C1b domain</td>
</tr>
<tr>
<td>Bp_9LM_r</td>
<td>ACTTACATCTATGGGTACATTG</td>
<td>AA residue 9 L→M</td>
</tr>
<tr>
<td>Bn_9IL_f</td>
<td>TCACAATTTCCTGGCCCTCACCTTC</td>
<td><em>B. neritina</em> C1b domain</td>
</tr>
<tr>
<td>Bn_9IL_r</td>
<td>GGTATGAAATCAGGTAGGAAC</td>
<td>AA residue 9 I→L</td>
</tr>
<tr>
<td>Bn_41RK_f</td>
<td>TGTCACAAGAATGTGAGAAGCTATGCCC</td>
<td><em>B. neritina</em> C1b domain</td>
</tr>
<tr>
<td>Bn_41RK_r</td>
<td>GTTGTGACCACATGCTTC</td>
<td>AA residue 41 R→K</td>
</tr>
<tr>
<td>Bn_5PV_f</td>
<td>CCGATTCAAAGTGCCACATTTATTCGGG</td>
<td><em>B. neritina</em> C1b domain</td>
</tr>
<tr>
<td>Bn_5PV_r</td>
<td>TGAGGAACATTTTATTCCC</td>
<td>AA residue 5 P→V</td>
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<tr>
<td>Bn_9IL_12HT_f</td>
<td>CACTCTCTGAGACCACCTGCAGG</td>
<td><em>B. neritina</em> C1b domain</td>
</tr>
<tr>
<td>Bn_9IL_12HT_r</td>
<td>CCCAGAAAATTGTGAGGTTTGAAATCGG</td>
<td>AA residue 9 I→L 12 H→T</td>
</tr>
<tr>
<td>Bn_8FY_f</td>
<td>CCTCACAATTATATGCGGCTTC</td>
<td><em>B. neritina</em> C1b domain</td>
</tr>
<tr>
<td>Bn_8FY_r</td>
<td>TTTGAATCGGTAGGAAC</td>
<td>AA residue 8 F→Y</td>
</tr>
<tr>
<td>Bn_9IM_f</td>
<td>ACAATTTTATGCGGCTACCTTC</td>
<td><em>B. neritina</em> C1b domain</td>
</tr>
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<td>Bn_9IM_r</td>
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<td>AA residue 9 I→M</td>
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<tr>
<td>Bn_12HT_f</td>
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<td><em>B. neritina</em> C1b domain</td>
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<td>Bn_12HT_r</td>
<td>AAATTGAGGCTGATGCG</td>
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<td>Bn_9IM_12HT_f</td>
<td>CTACCTCTGTCGACCACCTGCAGG</td>
<td><em>B. neritina</em> C1b domain</td>
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<tr>
<td>Bn_9IM_12HT_r</td>
<td>GCCCCATAAAATTGTGAGGTTTGAAATCGG</td>
<td>AA residue 9 I→M 12 H→T</td>
</tr>
</tbody>
</table>

Methylated template DNA was then removed via *DpnI* digestion at 25°C for 5 minutes.

Resulting products were transformed into transformed into chemically competent *E. coli* XL1-
Blue (Stratagene) cells, plated on LB agar with 50 µg/mL KAN, and incubated at 37°C overnight. Resulting colonies were screened for insert by colony PCR using T7 and T7 terminator primers and One Taq Polymerase (NEB). Construct plasmids that were found to have
an insert size of interest were extracted using Zyppy™ Plasmid Miniprep Kit (Zymo), column
purified, and quantified using Nandrop. Purified plasmids were sequenced at the DNA Analysis
Facility at Yale University, New Haven, CT, USA. Substituted amino acid residues PKC C1b
construct sequences were analyzed by C1b domain sequence comparison.

4.2.3 Overexpression

4.2.3.1 Cell Growth and Induction

Bugula neritina PKC δ C1b (pKBbn2512), Bugula pacifica PKC δ C1b (pKBbp311),
Rattus norvegicus PKC δ C1b (pKBrt2511), and B. neritina PKC δ C1b substitution mutant
(pKBbn2512_H12T) plasmids were transformed in chemically competent E. coli BL21
(Novagen) cells. Plasmids were initially grown in 50 mL tubes with 10 mL of Luria Bertani-
Miller broth (LB) with 50 µg/mL KAN overnight at 37°C shaking at 250 rpm. A second pass of
the cells were grown in 2 liter flasks with 1.5 liters of terrific broth (TB) with 5% glycerol
supplemented with 50 µg/mL KAN at 25°C with shaking at 200 rpm. Cell densities were
measured every hour until cells reached optical density (OD_{600}) of 1. Flasks were then incubated
at 16°C for 1 hour to acclimate cells to the lower temperature. BL21 cells were induced with
100mM IPTG and incubated shaking at 200 rpm at 16°C for 24 hours.

4.2.3.2 Extraction of Recombinant PKC C1 domain proteins

Each construct’s cells were centrifuged at 4,000 rpm at 4 °C for 25 minutes. The protein
was extracted using BugBuster® (Merck Millipore, Darmstadt, Germany) reagent specifications.
BugBuster® was diluted 1:10 with QIAexpressionist (Qiagen, Valencia, California, USA) lysis
buffer pH 8 consisting of 50mM sodium phosphate (monobasic), 300mM sodium chloride, and
10mM imidazole. Cells were thoroughly resuspended in the 1X Bugbuster® and incubated with
slight agitation for 1 hour at room temperature. The soluble and insoluble protein fractions were separated by centrifugation at 4,000 rpm for 40 minutes.

4.2.4 Purification of PKC C1b domains

4.2.4.1 Ni-NTA purification

The target proteins were purified from the soluble fractions with Ni-NTA chromatography. Ni-NTA chromatography was performed by mixing the soluble protein fraction with Ni-NTA agarose (Qiagen) and incubating samples at 4°C with slight agitation for 1 hour. Protein samples were added to a column where cell lysate was removed by gravity flow. The column was washed twice with wash buffer pH 8 consisting of 50 mM sodium phosphate, 300mM sodium chloride, and 20mM imidazole. Protein was eluted from the column using elution buffer pH 8 consisting of 50 mM sodium phosphate, 300mM sodium chloride, and 250mM imidazole. Protein fractions were separated using SDS-PAGE.

4.2.4.2 Thrombin Cleavage of 6X His-tag from BryAT1

pKBbn2512, pKBbn2512_H12T, pKBbp311, and pKBrt2511 were concentrated using Amicon® Ultra-4 centrifugal filters (Merck Millipore Ltd.) for 30 minutes at 4,000 rpm at 4°C. All proteins were exchanged into calcium chloride buffer consisting of 50mM Tris-HCl, pH 8, and 10 mM calcium chloride by centrifuging at 4,000 rpm for 30 minutes at 4°C in Amicon® Ultra-4 filter. Thrombin cleavage was performed according Thrombin CleanCleave™ Kit (Sigma-Aldrich) protocol at room temperature with gentle agitation for 4 hours. Proteins were recovered by supernatant collection using room temperature centrifugation at 2,500 rpm for 5 minutes.
4.2.4.3 Size-Exclusion Chromatography

pKBbn2512, pKBbn2512_H12T, pKBbp311, and pKBrt2511 were purified with size-exclusion chromatography (SEC) using a fractionation range of 3000–7000 Da. Column was packed using 15 mL of Superdex® 75 agarose beads (Sigma-Aldrich, St. Louis, Missouri, USA) in a XK16/20 (GE, Uppsala, Sweden) glass column. SEC purification running buffer consisted of 0.2µm filtered 50mM monobasic sodium phosphate, 50mM dibasic sodium phosphate, and 150mM sodium chloride. Purification was performed on ÄKTA explorer 100 (GE Healthcare, Malbourgh, MA, USA) at 0.4 mL/min flow rate for approximately 1 hour. All protein fractions were visualized using SDS-Page to ensure removal of unwanted proteins.

4.2.4.4 Concentration and Quantification

Protein fractions with band of interest were concentrated using Amicon® Ultra-4 centrifugal filters for 30 minutes at 4,000 rpm at 4°C. Buffer exchange was performed using HBS-P + 0.1% DMSO buffer consisting of 10mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) pH 7.4, 150mM sodium chloride, 0.05% Tween 20, and 0.1% DMSO by centrifugation for 40 minutes at 4,000 rpm at 4°C. Concentrated proteins were filtered with 0.22 µm low protein binding Durapore® (PVDF) membrane (Merck Millipore Ltd.). Protein concentrations were quantified by Pierce™ bicinchoninic acid (BCA) assay (Pierce Biotechnology) using bovine serum albumin (BSA) as a standard.
4.2.5 SPR: Binding affinity of bryostatin compounds to C1b domains

4.2.5.1 Chip, Buffers, Protocol

Binding affinities of constructs pKBbn2512, pKBbp311, pKBrt2511, and pKBbn2512_H12T with bryostatin-1 and phorbol 12-myristate 13-acetate (PMA) were quantified using surface plasmon resonance (SPR). SPR experiments were performed with pKBbn2512, pKBbp311, pKBrt2511, and pKBbn2512_H12T affixed to Series S Sensor Chip CM5 (GE Healthcare) via the amine coupling. The NTA sensor chip consists of a gold metal film on one side, and the other side is a carboxymethylated dextran that irreversibly immobilizes molecules via -NH2, -SH, -CHO, -OH, or -COOH groups. The gold metal film is exposed to a polarized light source causing the excitation of electrons on the gold metal surface, resulting in surface plasmons. The PKC δ C1b domains, ligands, are tethered to the chip and will be exposed to the analytes of bryostatin-1 and PMA. Binding associations (k_a) and disassociations (k_d) of ligands to analyte are provided in a sensorgram data consisting of data points of RU plotted versus time (seconds). The affinity constant K_D is calculated by the ratio of rate constants k_a and k_d (K_D = k_d / k_a). The K_d was measured at multiple analyte concentrations ranging between 0.1 and 10 times the K_d of the interaction. Bryostatin-1 was resuspended in 0.1% DMSO and diluted to a concentration of 55 μM. PMA was resuspended in 100% DMSO and diluted to a concentration of 50 μM in 0.1% DMSO.

Surface plasmon resonance was performed on a Biacore T-200 (GE Healthcare, Uppsala, Sweden) instrument using two Series S Sensor Chip CM5 (Lot # 10252793 and Lot # 1009028, GE Healthcare, Uppsala, Sweden). The experimental running buffer consisted of 0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% Tween 20, and 0.1% Dimethyl sulfoxide (DMSO).

Approximately 30 μg / mL of pKBbn2512, pKBbp311, pKBrt2511, and pKBbn2512_H12T was...
bound to the CM5 chips via amine coupling 20 μM N-hydroxysuccinimide (NHS) / 5 μM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Amine Coupling Kit, GE Healthcare, Uppsala, Sweden) for 7 min at flow rate of 5 μl/min and excess cross linkers were quenched by injection of 1 M ethanolamine for 10 min.

Single cycle kinetics were measured at 10 hertz (Hz) with a 30 μL/min flow rate for a 30 second association time and a 30 second dissociation time at 25°C. Bryostatin-1 and PMA were diluted in HBS-P + 0.1% DMSO to achieve experimental analyte concentrations of 0 μM, 0.625 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM, 12.5 μM, and 15 μM. Due to bryostatin-1’s sensitivity to UV light, fresh analyte concentrations were made for each experimental SPR run. Response units and time were evaluated using Biacore T200 Software v3.1 with parameters of 4 seconds before injection stop and 5 seconds after with a steady state curve fit. Channel one of each CM5 chip was used as a reference blank to subtract background to achieve a baseline for accurate $K_D$ calculations. DMSO correction curves were utilized to extrapolate the background caused by the DMSO non-specific binding. For each experiment, a new correction curve concentrations were prepared by diluting 100% DMSO to 2% using HBS-P + 0.1 % DMSO then serially diluted to 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, and 3.125 nM concentrations.

4.3 Results

4.3.1 Identification of PKC C1b δ domains

PCR amplification of $B. turrita$, $B. turbinata$, $B. simplex$, $B. pacifica$, and $B. stolonifera$ using the primers proved challenging. Across the Bugula species and PKC isoforms primer sets analyzed, only $B. pacifica$ PKC δ C1b domain amplified with a C1b domain sequence that differed from that of $B. neritina$. $Rattus norvegicus$ PKC δ C1b domain was also amplified successfully.
4.3.2 Construction and Purification of PKC δ C1b domains

*B. neritina*, *B. neritina* H12T, *B. pacifica*, and *R. norvegicus* PKC δ C1b domains were all successfully ligated into pET28b vectors. Protein overexpression in BL21 cells and protein

![Image of SDS-page](image_url)

Figure 4.2: SDS-Page of Purified PKC δ C1b Domain
Overexpressed purified proteins of *B. neritina* PKC δ C1b domain (pKBbn2512, ~10.7kDa), *B. pacifica* PKC δ C1b domain (pKBbp311, ~10.7 kDa), *R. norvegicus* (pKBrt2511, ~10.7 kDa), and *B. neritina* H12T (pKB2512_H12T, ~10.7 kDa) with NEB (10-250 kDa) protein ladder
puration procedures were also successful producing adequate protein concentrations for future SPR experiments (Figure 4.2). Protein concentration for pKB114 was 629.3 µg/mL while pKBbn2512_H12T was 529.1 µg/mL. pKBrt2511 had the highest concentration at 1943.4 µg/mL and pKBbp311 was lowest at 242.4 µg/mL.

4.3.3 Binding Affinities for PKC δ C1b Domains to Bryostatin-1

Surface density of protein attachment to the CM5 Chip (Lot # 1009028) for pKBbn2512 was 1512 RU while pKBbp311 was 1470 RU and CM5 Chip (Lot # 10252793) had 1046.4 RU of pKBbn2512_H12T immobilized with 1764.2 RU of pKBrt2511. Data for the KD values obtained during the SPR experiments with bryostatin-1 as the analyte were plotted response (response units) versus concentration (M) (Figure 4.2 -4.5). KD raw data of binding affinities for each experiment were recorded (Table 4.3). KD values of PKC δ C1b domains from B. pacifica (pKBbp311), R. norvegicus (pKBrt2511), and B. neritina H12T were normalized to B. neritina (pKBbn2512) KD values. Normalized binding affinities values for B. pacifica ranged from 0.02 to 4.21 with an average of 1.30 and standard deviation of these values was 1.51. R. norvegicus normalized values ranged from 0.11 to 6.56 with an average of 2.81 and standard deviation of 2.66. B. neritina His12 to Thr12 substitution normalized KD values ranged from 0.44 to 4.76 with an average of 1.75 and a standard deviation of 1.56 (Table 4.4).
Figure 4.3: SPR Experiment of B. neritina PKC δ C1b domain (pKBbn2512) versus Bryostatin-1

![Graph](image1)

pKBbn2512 exposed to 0 µM, 0.625 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 12.5 µM, and 15 µM of bryostatin plotted with response units (RU) versus concentration in molarity (M). KD value of 5.917E-6.

Figure 4.4: SPR Experiment of B. pacifica PKC δ C1b domain (pKBbp311) versus Bryostatin-1

![Graph](image2)

pKBbp311 exposed to 0 µM, 0.625 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 12.5 µM, and 15 µM of bryostatin plotted with response units (RU) versus concentration in molarity (M). KD value of 3.77E-6.
Figure 4.5: Experiment of R. norvegicus PKC δ C1b domain (pKBrt2511) versus Bryostatin-1

pKBrt2511 exposed to 0 µM, 0.625 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 12.5 µM, and 15 µM of bryostatin plotted with response units (RU) versus concentration in molarity (M). KD value of 4.161E-6.

Figure 4.6: SPR Experiment of B. neritina PKC δ C1b domain H12T (pKBrt2512_H12T) versus Bryostatin-1

pKBbn2512_H12T exposed to 0 µM, 0.625 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 12.5 µM, and 15 µM of bryostatin plotted with response units (RU) versus concentration in molarity (M). KD value of 3.543E-6.
### Table 4.3: Binding Affinity of Bryostatin-1 to PKC δ C1b Constructs

<table>
<thead>
<tr>
<th>PKC δ C1b Construct vs Bryostatin-1 K&lt;sub&gt;D&lt;/sub&gt; Value</th>
<th>pKBbn2512</th>
<th>pKBbp311</th>
<th>pKBrt2511</th>
<th>pKBbn2512H&gt;T</th>
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<tbody>
<tr>
<td>2.02E-06</td>
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<td>3.54E-06</td>
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<td>1.52E-06</td>
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<td>1.43E-06</td>
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</tr>
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<td>6.34E-07</td>
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<td>4.16E-06</td>
<td>3.02E-06</td>
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</tr>
<tr>
<td>1.09E-05</td>
<td>3.77E-06</td>
<td>1.21E-06</td>
<td>4.77E-06</td>
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</tr>
</tbody>
</table>

### Table 4.4: Normalized Binding Affinities of Bryostatin-1 and PKC C1b Domains

<table>
<thead>
<tr>
<th>Binding Affinities Normalized to B. neritina</th>
<th>BP311</th>
<th>RT2511</th>
<th>BN2512:HT</th>
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<td>4.76</td>
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<td>0.35</td>
<td>0.11</td>
<td>0.44</td>
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<tr>
<td>Average Normalized</td>
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<td>2.81</td>
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<tr>
<td>Standard Deviation</td>
<td>1.51</td>
<td>2.66</td>
<td>1.56</td>
</tr>
</tbody>
</table>
4.3.4 Binding Affinities for PKC δ C1b Domains to PMA

Response units (RU) of pKBbn2512, pKBbp311, pKBrt2511, and pKBbn2512_H12T and PMA interactions were plotted response against analyte concentrations (M) to obtain binding affinities, $K_D$ values. (Figure 4.6 - 4.9). $K_D$ raw data of binding affinities for each experiment were recorded (Table 4.5). $K_D$ values of PKC δ C1b domain binding affinity from *B. pacifica* (pKBbp311), *R. norvegicus* (pKBrt2511), and *B. neritina* H12T to PMA were normalized to *B. neritina* (pKBbn2512) PMA $K_D$ values. Normalized binding affinities values for *B. pacifica* ranged from 0.02 to 3.38 with an average of 1.01 and standard deviation of these values was 1.25. *R. norvegicus* normalized values ranged from 0.61 to 5.02 with an average of 2.25 and standard deviation of 1.54. *B. neritina* His12 to Thr12 substitution normalized $K_D$ values ranged from 0.10 to 1.12 with an average of 0.49 and a standard deviation of 0.37 (Table 4.4).

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**Figure 4.7: SPR Experiment of B. neritina PKC δ C1b domain (pKBbn2512) versus PMA**

![Graph](image)

pKBbn2512 exposed to 0 µM, 0.625 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 12.5 µM, and 15 µM of PMA plotted with response units (RU) versus concentration in molarity (M). KD value of 1.17E-6.
**Figure 4.8: SPR Experiment of *B. pacifica* PKC δ C1b domain (pKBbp311) versus PMA**

pKBbp311 exposed to 0 µM, 0.625 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 12.5 µM, and 15 µM of PMA plotted with response units (RU) versus concentration in molarity (M). KD value of 1.78E-6.

**Figure 4.9: SPR Experiment of *R. norvegicus* PKC δ C1b domain (pKBrt2511) versus PMA**

pKBrt2511 exposed to 0 µM, 0.625 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 12.5 µM, and 15 µM of PMA plotted with response units (RU) versus concentration in molarity (M). KD value of 1.78E-6.
Table 4.5: Binding Affinity of PMA to PKC δ C1b Constructs

<table>
<thead>
<tr>
<th>PKC δ C1b Construct vs PMA K&lt;sub&gt;D&lt;/sub&gt; Value</th>
<th>pKBbn2512</th>
<th>pKBbp311</th>
<th>pKBrt2511</th>
<th>pKBbn2512_H12T</th>
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Table 4.6: Binding Affinities of PMA Experiments Normalized to B. neritina

<table>
<thead>
<tr>
<th></th>
<th>BP311</th>
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<th>BN2512:HT</th>
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<tr>
<td></td>
<td>1.25</td>
<td>1.54</td>
<td>0.37</td>
</tr>
</tbody>
</table>

4.4 Discussion

A general rule of binding affinity is that the lower the $K_D$ value the higher the affinity for the ligand. Dissociation constant ($K_D$) values represent the point where the ligand, PKC C1b domains, and analyte, bryostatin-1 or PMA, molecules are bound to ligand at equilibrium (half bound and half unbound). Binding affinities are values taken at a specific time duration the experiments. Saturation levels of ligand-analyte binding will vary according to the strength at which the interaction occurs. Not only does the time at which the changes in light occur effect affinities, pH, temperature, and buffer can vary the results of any experiment (Kuriyan, 2008). Bryostatin-1 and PMA are small molecules which are soluble in DMSO. DMSO concentrations were kept as low as possible at 0.1% and were consistent throughout samples and buffers; however, DMSO can alter the affinity due to non-specific binding to the chip surface. In order to counteract this issue, correction curves were used in each experiment to subtract the background
caused by the DMSO. Surface density of the attached protein to the chip channel can also play role in the variation of light refraction and subsequent affinity readings. Immobilized protein concentrations were kept uniform at 30 ng/µL for surface density consistency since $K_D$ is a measure of protein molecules that are bound to the analyte.

Binding affinities of the PKC δ c1b domains to bryostatin-1 and PMA showed variation across the constructs. On average, *B. pacifica* C1b domain had a binding affinity to bryostatin-1 that was very close to of *B. neritina* in both bryostatin-1 and PMA experiments. *R. novergicus* had noteworthy differences in $K_D$ values from *B. neritina* with normalized averages of 2.81 for bryostatin-1 and 2.25 with PMA. *B. neritina* H12T had average normalized binding of 1.75, while higher than *B. pacifica*, showed no major difference from that of *B. neritina* bryostatins under the conditions of this experiment. The average binding affinity of PMA to pKBbn2512_H12T had the smallest margin of difference from pKBbn2512, but it still was not as notable of a difference to that of *B. pacifica*. The amino acid substitution of the threonine for the histidine at position 12 in *B. neritina* did make the construct more similar to *B. pacifica* in sequence; however, it did not majorly alter the binding affinity in bryostatin-1 or PMA experiments to resemble *B. pacifica* C1b domain more than pKBbn2512 under these conditions. Due to this data, it is possible that the histidine residue at position 12 is not involved in the ability of bryostatin-1 to activate PKCs. While *R. norvegicus* and *B. pacifica* have never been exposed to the bryostatin compound naturally and only *R. norvegicus* had a meaning change in to the bryostatin-1 than that of *B. neritina*.

### 4.5 Conclusion

The previous exposure of *B. neritina* to bryostatin compounds via symbiont could have caused an evolutionary change in the host PKCs. Notable differences in ligand-*B. neritina* C1b...
binding affinity of versus *R. norvegicus* that has not had the same exposure could suggest a
certain amount of coevolution. No remarkable changes in the binding affinity of
pKBbn251_H12T to bryostatin-1 versus *B. neritina* could mean that the substitution at position
12 to a threonine might not be involved in bryostatin affinity to PKCs. Continued work on amino
acid substitutions in *B. neritina* could provide more evidence about which amino acids are
involved in PKC activation. Also, more *Bugula* spp. PKC C1b domains should be identified
across the conventional and novel isoforms, and cloned with protein expression to further test the
effects that bryostatin-1 can have on C1b domains. Binding affinity differences between the
*Bugula* spp. with and without natural bryostatins could lead to a better understanding of where
and how the bryostatin-1 interacts with C1b domains for PKC activations.
5 CONCLUSIONS

5.1 Summary

Symbiotic relationships can be classified in many ways, from mutualistic to parasitic. In many cases, the symbiont biosynthesizes natural products that can be utilized by the host. For *B. neritina*, the symbiont *E. sertula* synthesizes bryostatins using an unusual trans-AT PKS system. The mechanism by which the trans-AT recognizes the correct enzymatic domain for formation of the bryostatin polyketide is not fully characterized. The research of protein-protein interactions between trans-AT and the KS-AT interdomain regions has determined the potential PKS KS-AT region of the module location as where trans-AT interaction occurs.

The bioactive bryostatins may also play a role in the host-symbiont interaction. Understanding how the host adapts to symbiont-produced natural products will illustrate another mechanism of host-symbiont co-evolution. Bryostatins are useful in deterring the predation of *B. neritina* larvae, but are potent PKC activators via C1b domain binding. In this study, I determined binding affinities of bryostatin-1 and PMA compounds to PKC C1b domains from *B. neritina* and other *Bugula* spp. to establish the relative interaction with each *Bugula* spp. to bryostatins. These data can advance knowledge of how the *Bugula* host has potentially adapted to the symbiont-produced bryostatins over time.


References


