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Sources of Diversity in Bactobolin Biosynthesis by *Burkholderia thailandensis* E264

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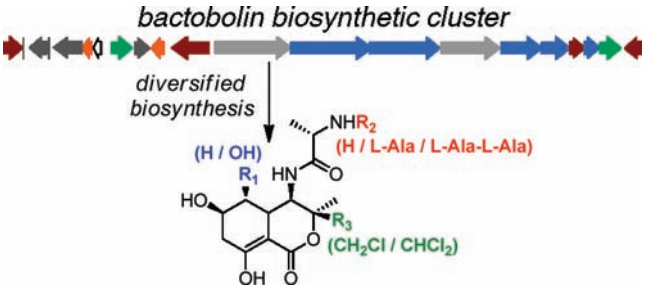
ABSTRACT



A series of deletion mutants in the recently identified bactobolin biosynthetic pathway defined the roles of several key biosynthetic enzymes and showed how promiscuity in three enzyme systems allows this cluster to produce multiple products. Studies on the deletion mutants also led to four new bactobolin analogs that provide additional structure–activity relationships for this interesting antibiotic family.

The proteobacterial genus *Burkholderia* contains significant plant and animal pathogens including *B. pseudomallei* and *B. mallei*, the causative agents of meliodosis (Whitmore disease) in humans and glanders in horses.[1] *B. thailandensis*, a nonvirulent member of the genus, has become a model organism in which to study factors controlling both virulence and the expression of secondary metabolites.[2] The completed genome sequence of *B. thailandensis* contains at least a dozen polyketide synthase (PKS) and/or nonribosomal peptide synthetase (NRPS) biosynthetic clusters, but only three of these clusters have been associated with their corresponding small molecule products.[3] This report describes combined genetic and metabolomic studies that refine our understanding of the biosynthesis of members of the bactobolin antibiotic family in *B. thailandensis*.

Our interest in the bactobolins originated in studies by Duerkop et al. showing that *N*-acylhomoserine lactones, the typical quorum sensing signals in proteobacteria,[4] initiate the synthesis of a potent antibiotic.[5] This study also identified the biosynthetic locus (*bta*) of bactobolin. Later work by Seyedsayamdost et al. identified the products of the *bta* cluster as bactobolins (Figure 1, **1**–**4**) and provided a clearer picture of the *bta* biosynthetic locus (Figure 2A) as well as a tentative model for bactobolin biosynthesis (Figure 2B).[3c]

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In this model, the bactobolin core is built using NRPS and PKS modules to link substrates and an aldol condensation to close the carbocyclic ring (Figure 2B).[3c] BtaK, an NRPS with an Ala-specific adenylation (A) domain, generates a dialanyl thioester, and BtaN, also an NRPS, adds an 3-hydroxy-4,4-dichlorovaline group (OH-Cl_2-Val), which is synthesized by the dichlorinase BtaC, and hydroxylated by either BtaA or BtaU, putative Fe-dependent hydroxylases.[6] These enzymes may use BtaB and/or BtaD, which are free-standing thiolation (T) domains for substrate tethering (Figure 2A). The resulting tripeptide (**10**) is transferred to a PKS assembly line, where BtaOML then adds three acetyl units derived from malonyl CoA, and BtaL likely catalyzes an aldol condensation followed by reduction of the resulting unsaturated ketone **11**. Finally, either BtaS, a putative type II thioesterase, or BtaP, a predicted β-lactamase,[7] catalyzes lactone formation to produce **4**.

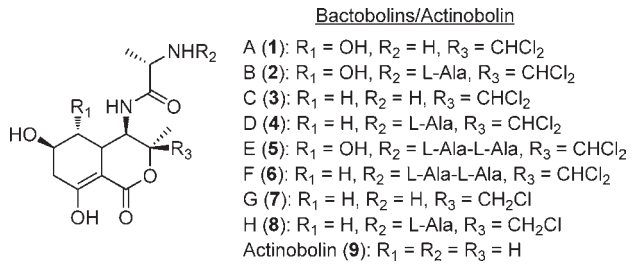

Figure 1. Structures of bactobolins A−H and actinobolin.

This model has several ambiguities, and in an effort to further refine it, four markerless deletion strains—BtaB, BtaL's short chain dehydrogenase (SDR) domain, BtaP, and BtaU (Figure 2A)—of *B. thailandensis* were constructed using methods previously reported.[8]

BtaB and BtaD have a sequence similarity to standalone T domains, though the former has a modified signature sequence for this class of enzymes.[9] The BtaB deletion mutant was generated to identify which of these T domains is used in bactobolin production as this knowledge would facilitate future *in vitro* studies of the enzymes involved in valine modification. The metabolic profile of the BtaB mutant was very similar to that of wt *B. thailandensis*. It produced all four bactobolins with yields similar to wt (Figure S1, Supporting Information). This result and the predictions based on sequence analysis both argue that BtaD is likely the active T domain in valine modification (Figure 2).

BtaL contains a short chain dehydrogenase (SDR) domain that was the most likely candidate to carry out reduction of the aldol condensation product (**11**).[10] The SDR domain in BtaL was deleted using the identifiable linker regions between the AT and ACP domains in BtaL (Figure 2A) with the optimistic expectation that this mutant would allow intermediates to accumulate that could be structurally characterized. However, the SDR deletion in BtaL completely abrogated bactobolin production and metabolomic analysis showed no small molecules with the isotopic signature for one or two Cl atoms.

BtaP seemed likely to be involved in lactone formation.[7] Its deletion, like the BtaL SDR domain deletion strain, also completely abrogated bactobolin production (data not shown). The results from the BtaL and BtaP deletion strains are consistent with both playing important roles in bactobolin biosynthesis, as indicated by the model, but the lack of isolable intermediates prevents a definitive assignment of function. However, comparison between the BtaP/BtaL deletion strains and wt did reveal that the wt strain produced four other compounds with $[M+H]^+$ at m/z 333, 404, 509, and 525 (Figure S2, Supporting Information). Their isotopic distribution was consistent with the presence of chlorine atoms, and the retention times suggested that they were related to the known bactobolins. Thus, while the BtaL and BtaP mutants failed to accumulate biosynthetic intermediates, they revealed four unsuspected bactobolin analogs from wt *B. thailandensis*.

By sequence homology, BtaU was likely a Fe-dependent hydroxylase, but whether it installed the hydroxyl function in the modified valine or at C-5 of the bactobolin skeleton was unclear. In contrast to wt *B. thailandensis*, the BtaU mutant produced only the nonhydroxylated analogs **3** and **4**, but not **1** and **2** (Figures 1 and 3A), indicating that BtaU is the bactobolin hydroxylase (Figure 3B). Together, the BtaB and BtaU deletion studies allow us to refine our model for the biosynthesis of OH-Cl_2-Val and OH-Cl-Val (see below), in which BtaD is the active T domain, BtaC the chlorinase, and BtaA the likely hydroxylase (Figure 3C).[11,12]

We next investigated the four new bactobolins produced by wt *B. thailandensis*. The peaks in the HPLC-MS profile at m/z 525 and 509 were consistent with the addition of an extra alanine residue to **2** and **4**, leading to the suspicion that these compounds were the corresponding trialanine derivatives, **5** and **6**. The isotopic distribution of the m/z 333 and 404 peaks suggested that they contained a single chlorine atom rather than the two chlorine atoms of all previously known bactobolins. These masses were consistent with the replacement of a chlorine atom from bactobolins C (**3**) and D (**4**) with a hydrogen atom, leading to

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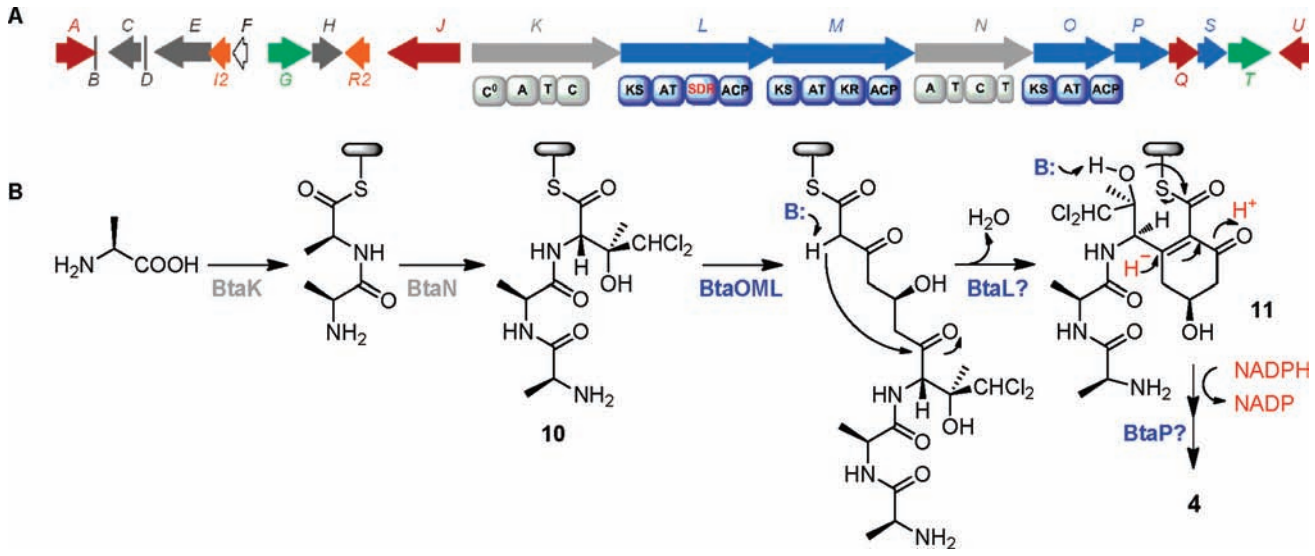

Figure 2. Bactobolin gene cluster and a proposed biosynthetic model. (A) Schematic of the bactobolin gene cluster. Genes involved in regulation (orange), metabolite/product transport (green), synthesis of OH-Cl_2-Val (dark gray), synthesis of Ala-Ala (light gray), synthesis of the C_6-polyketide and ensuing reactions (blue), tailoring reactions (red), and genes of unknown function (white) are detailed. The domain architectures for the PKS and NRPS genes are also shown. (B) Proposed bactobolin biosynthesis.[3c] See text for a description.

their tentative identification as the dechloro analogs **7** and **8** (Figure 1).

In order to confirm the structures of **5**–**8**, production cultures of *B. thailandensis* E264 were grown in a liquid LB medium for 24 h at 30 °C. The spent medium was fractionated using HP-20 and Hypercarb chromatography and reversed-phase HPLC (Supporting Information) to give the new analogs **5**–**8**, along with the known bactobolins **1**–**4**. Analysis of the HR-ESI-MS(+) and 1D and 2D NMR data for **5**–**8** (Tables S1–S4, Figures S3–S6, Supporting Information) confirmed their predicted structures (Figure 1). Comparison of the NMR data for **5**–**8** with the NMR data for **1**–**4** also confirmed that they have the same relative configuration. The absolute configurations of the alanine residues in **5**–**8** were determined by acid hydrolysis followed by Marfey's analysis[13] and indicated L-alanine in each case.

The presence of the trialanine bactobolin analogs **5** and **6** suggests that the N-terminal C^0-domain in BtaK is active, or that the module generated by the C-domain in BtaK and the N-terminal AT domains in BtaN, which is responsible for inserting the second alanine, acts iteratively to introduce a third Ala residue (Figure 2A).[3c] Such domain and module reuse, and skipping, has been documented in several modular NRPS systems recently.[14] We were unable to see any evidence for the corresponding tetra (or higher)-peptides in the HPLC-MS profile. Once generated, the putative oligopeptidase (BtaJ) may act on the trialanine bactobolins to the give corresponding mono- and dialanine derivatives.[15] This reaction may be preceded by acetylation of the N-terminal alanine via the putative acetyltransferase BtaQ, as previously proposed.[3c,15] Biochemical studies will be necessary to distinguish between the mechanisms above.

Compounds **7** and **8** contain the unnatural amino acid 3-hydroxy-4-chlorovaline (Cl-Val) in place of 3-hydroxy-4,4-dichlorovaline (Cl_2-Val). The structures of the monochlorinated bactobolin analogs **7** and **8** have implications for the specificity of the bactobolin hydroxylase, BtaU, that incorporates the hydroxyl group at C-5 in **1** and **2** (Figure 3B). Among the known dichlorinated bactobolins, the major analogs (**1** and **2**) contain a hydroxyl group at this position, while the minor analogs (**3** and **4**) do not. However, neither of the monochlorinated bactobolins that could be detected (**7** and **8**) contained a hydroxyl group at this position. These results suggest that the hydroxylase enzyme BtaU may be specific for a dichlorinated bactobolin substrate.

The studies above show that the bactobolin biosynthetic cluster produces a small family of bactobolins, and the

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sources of this diversity lie in the variable number of alanine residues incorporated (BtaK or BtaN), the number of chlorine substituents on valine (BtaC), and the hydroxylation at C-5 (BtaU).

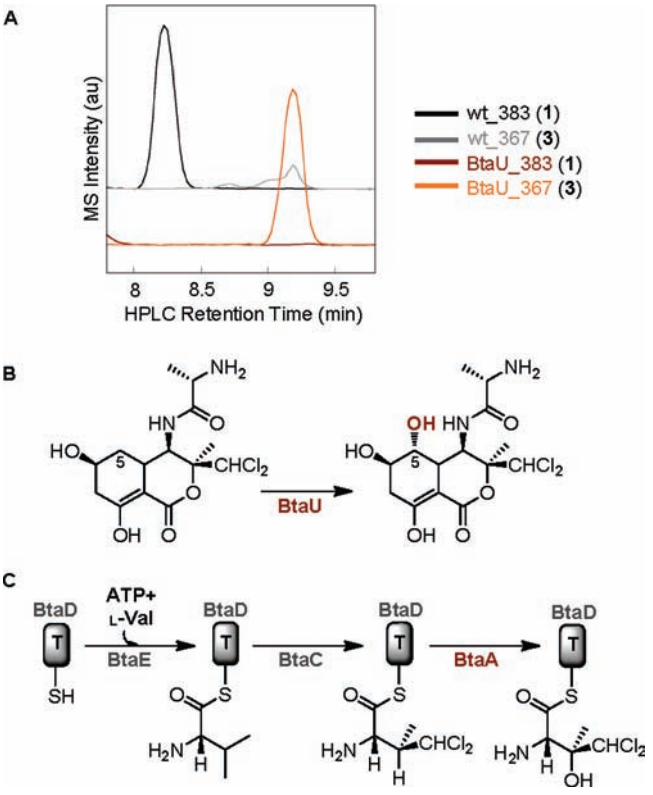

Figure 3. Metabolic profile of ΔBtaU *B. thailandensis*. (A) Ion-extracted HPLC-MS trace of partially purified culture supernatant from wt *B. thailandensis* (black trace for **1**, gray trace for **3**) and ΔBtaU *B. thailandensis* (brown trace for **1**, orange trace for **3**). (B) Putative reaction catalyzed by BtaU. (C) Proposed biosynthesis of OH-Cl_2-Val based on the results herein. OH-Cl-Val is made in an analogous fashion, where BtaC adds a single Cl. The order of reactions by BtaC and BtaA is not known.

To examine the structure–activity relationships for the bactobolin family (**1**–**8**), they were tested against *B. subtilis* and *E. coli*. Interestingly, **3** and **7** displayed similarly weak activities (minimal inhibitory concentration, MIC, of 50 μg/mL vs *B. subtilis*) as did **4** and **8** (Supporting Information, Figure S7) indicating that the second chlorine atom has little effect on the antibiotic activity of these compounds (Figure 4). In contrast, **5** and **6** were inactive, as expected based on the potency of **4** and **2**.[3c] Similar assays with **9** indicate that it is 20-fold less potent than **1**.[16] The difference is provided by the dichloromethyl group present in **1** and absent in **9**, in which threonine replaces the modified valine (Figure 1.).[17] The evolution of OH-Cl-Val and OH-Cl_2-Val incorporation in the *Burkholderia* lineage, rather than the threonine found in actinobolin, generated a more potent family of antibiotics. Hydroxylation by BtaU further increases the potency of **1** (MICs of 1.56 μg/mL vs *B. subtilis*) relative to those of **3** and **7** (Supplementary Table S5).

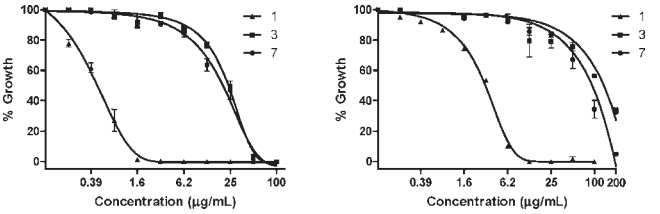

Figure 4. Inhibitory activity of **1**, **3**, and **7** against *B. subtilis* BS3610 (A) and *E. coli* K12 (B).

In summary, we have identified four new bactobolins, proposed functions for several important enzymes involved in bactobolin biosynthesis using gene deletion studies, and identified the structure-diversifying elements in bactobolin biosynthesis. Additional studies will be needed to completely define the intriguing steps involved in bactobolin production.

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Supporting Information Available. Experimental details, tables of NMR assignments and NMR spectra for **5**–**8**, metabolic profiles of wt and mutant *B. thailandensis* strains. This material is available free of charge via the Internet at http://pubs.acs.org.