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Quorum-Sensing-Regulated Bactobolin Production by *Burkholderia thailandensis* E264

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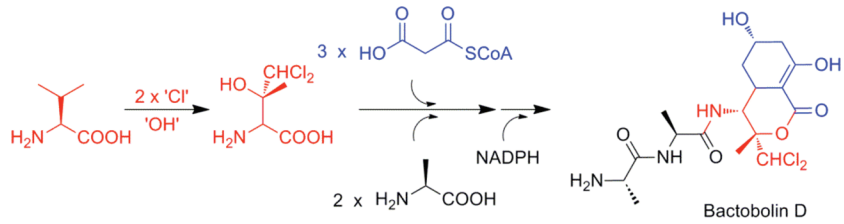
ABSTRACT



Bacterial acyl-homoserine lactones upregulated an uncharacterized gene cluster (*bta*) in *Burkholderia thailandensis* E264 to produce an uncharacterized polar antibiotic. The antibiotic is identified as a mixture of four bactobolins. Annotation of the *bta* cluster allows us to propose a biosynthetic scheme for bactobolin and reveals unusual enzymatic reactions for further study.

Sequenced bacterial genomes are appearing at an ever-increasing rate, and they document both the remarkable metabolic potential of these organisms and our limited ability to access this potential. The sequences reported thus far have revealed many more molecules whose existence can be inferred from genomic analysis than molecules whose existence has been established in the laboratory.[1] Many of these metabolites likely remain cryptic because their production is regulated, and the regulatory factors are not known. *Burkholderia thailandensis* E264, a Gram-negative bacillus that has become a model organism for its more virulent relatives, has a completely sequenced genome that contains at least 12 unassigned polyketide synthase (PKS) and/or non-ribosomal peptide synthase (NRPS) clusters, which are being extensively studied.[2] Many behaviors of Gram-negative bacteria are regulated in a population-density-dependent fashion by acyl-homoserine lactones (Ac-HSLs).[3] Recently, Duerkop et al. examined the regulation of antibiotic production in *B. thailandensis* E264 by Ac-HSL and showed that the two Ac-HSLs produced by the BtaR2-BtaI2 signaling system regulated the production of a polar antibiotic of unknown structure.[4] Using mutational analysis, they were

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able to define the biosynthetic locus (*bta* cluster) of the antibiotic.[4] Herein, we report the structure of the antibiotic and establish its activity against selected bacterial pathogens. Annotation of the biosynthetic cluster allows us to propose a biosynthetic scheme that serves as a starting point for examining the unusual reactions involved.

To isolate the antibiotic, *B. thailandensis* was cultivated as previously reported.[4] The antibiotic's highly polar, amphoteric nature and its optimal production in rich media (LB) necessitated unusual chromatographic steps (see Supporting Information). Using activity-guided fractionation against *B. subtilis* 3610, four active compounds were purified, and their structures were elucidated by 1-D/2-D NMR (Figures S1−S6 and Tables S1−S4 in Supporting Information) HR-MS, and MS/MS (Table S5) methods. The antibiotics belong to the bactobolin/actinobolin family (Figure 1).[5] The structures of

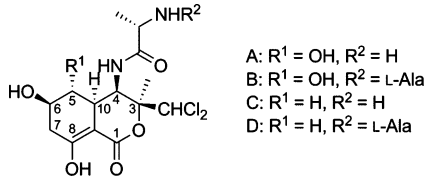

Figure 1. Bactobolins characterized in this work.

bactobolins A−C have previously been characterized by X-ray crystallography and chemical analysis.[6,7] Bactobolin D is new and likely a biosynthetic precursor to bactobolins A−C. The stereochemistry is based on $[\alpha]_D$ comparisons (Table S5), *J* coupling, and ROESY spectral analyses, which confirm the axial-axial relationship of protons H_5/H_{10} and H_5/H_6, the axial-equatorial relationship of H_{10}/H_4, and the homoallylic coupling between H_{10} and H_{7ax} (Figures S1−S6).[5,6] The stereochemistry of the Ala residue(s) was confirmed by acid hydrolysis followed by Marfey's analysis (see Supporting Information).[8]

Bactobolins have several interesting structural features. As suggested by the *bta* cluster, they are NRPS-PKS hybrids containing a di- or tripeptide fused to a C6-polyketide. The fusion occurs at the carboxyl group of 3-hydroxy-4,4-dichlorovaline (OH-Cl_2-Val), an unnatural amino acid with unknown biosynthesis that is thus far limited to the bactobolins. The enol lactone likely mimics a carboxyl group, allowing bactobolins to enter the cell via di- and tripeptide transporters.[9] Bactobolins differ from actinobolin, an antibiotic identified from *Streptomyces griseoviridis*, in that the OH-Cl_2-Val residue is replaced by Thr.[5a] This switch renders actinobolin 21-fold, 1000-fold, and 900-fold *less* active than bactobolin in *E. coli* kill assays, in vitro inhibition of translation, and murine antitumor activity, respectively.[10]

The identification of bactobolin and its link to the *bta* cluster allows us to propose a scheme for its biosynthesis. The modular 44 kb PKS/NRPS cluster is highly fragmented with product(s) inconsistent with the co-linearity rule (Figure 2A). On the basis of short intergenic sequences and a single identifiable Shine-Dalgarno sequence, the translation of *btaK-btaS* appears to occur from one polycistronic mRNA. The cluster contains six distinct elements: genes involved in regulation (orange), metabolite/product transporters (green), synthesis of OH-Cl_2-Val (gray), Ala-Ala (light gray) and the C6 polyketide and related reactions (blue), tailoring reactions (brown), and genes of unknown function (white, Figure 2A). Results of homology searches and other bioinformatic analyses (Table S6) lead us to propose the model in Figure 2B.

Annotation of the *bta* cluster provides a biosynthetic route for OH-Cl_2-Val and its insertion into the NRPS/PKS transfer line (Figure 2B, inset). Its biosynthesis requires both dichlorination and hydroxylation reactions. Dichlorination likely involves BtaC, a Fe-dependent chlorinase (57% identical to CytC3). BtaC contains an Ala residue, typical for Fe-dependent chlorinases, in place of the carboxylate group that usually occupies the facial triad and coordinates Fe in α-KG-dependent oxygenases.[11] Hydroxylation of Val (or Cl_2-Val) could be catalyzed by either BtaA or BtaU, both non-heme Fe-dependent dioxygenases, or even by BtaC as recent studies have delineated the requirements for Cl versus OH insertion by these enzymes.[12] BtaC is flanked by two free-standing thiolation (T) domains, where BtaB lacks the typical GXDS(L/I) sequence motif and may be inactive.[13] Thus, BtaD is likely the active T domain and may be primed with Val by BtaE, a Val-specific, stand-alone adenylation (A) domain. BtaH contains a GXCX(G/S) motif and likely acts as an CmaE-like transacylase,[14] by shuttling the assembled OH-Cl_2-Val from one T domain to another within the NRPS assembly line (Figure 2B, inset).

BtaK and BtaN are NRPSs with the domain architecture shown in Figure 2B. The first condensation (C) domain in BtaK does not contain the conserved active site motif (HHX_3DG) and is likely inactive.[13] Its A domain bears higher homology to Gly-specific domains, but no Gly-bearing bactobolins have been detected experimentally. In contrast, the A domain of BtaN has an Ala-specific sequence. After formation of Ala-Ala (**1**, Figure 2B), the C-terminal T domain of BtaN likely accepts the activated OH-Cl_2-Val (see

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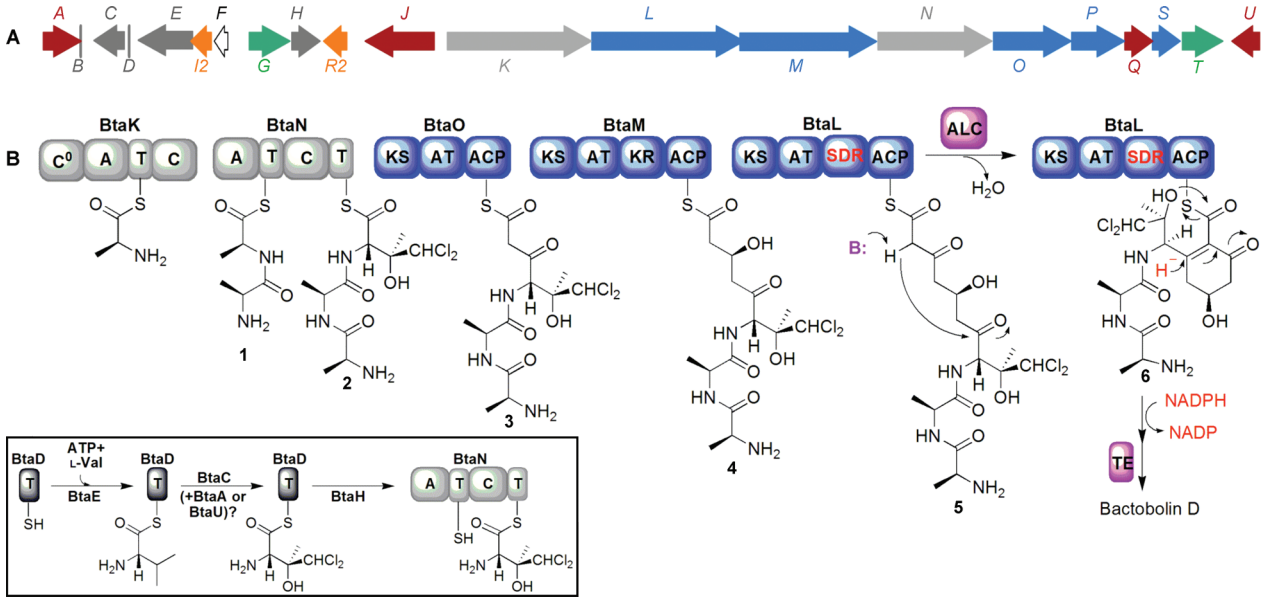

Figure 2. (A) Schematic of the bactobolin biosynthetic cluster detailing genes *btaA* through *btaU*. (B) Proposed biosynthetic scheme for bactobolin D. Note that the aldol condensation (ALC) and thioesterase (TE) reactions are not assigned to a specific protein or domain. See text for details. Inset, proposed biosynthesis of OH-Cl_2-Val and its subsequent transfer to the NRPS/PKS assembly line.

above), which the adjacent C domain may assemble into an Ala-Ala-(OH-Cl_2-Val) tripeptide (**2**). This is subsequently condensed to an acetyl group originating from malonyl CoA catalyzed by the ketosynthase (KS) domain of BtaO (**3**), followed by another acetyl group addition and ketoreduction (KR) by BtaM to give **4** (Figure 2B).

The final C2 unit may be added by BtaL, yielding **5**. BtaL contains a non-canonical KR domain that is similar to short chain dehydrogenases (SDR). There is a 300 amino acid region between the AT and SDR domains with no recognizable sequence homology. The ensuing plausible reactions that may yield bactobolin D, aldol condensation between OH-Cl_2-Val and the final malonyl-CoA unit and elimination of water to give **6**, followed by reduction of the resulting α,β-unsaturated ketone to generate the enol moiety (Figure 2B), lack clear precedents at this time. These reactions may be performed by the SDR domain in BtaL and the adjoining 300 amino acid region.[15,16] BtaS, a predicted type II thioesterase, may be involved in generation of the lactone. Alternatively, lactonization could be carried out by BtaP, a predicted β-lactamase, in accordance with recent studies by Awakawa et al.[17] Biochemical studies are in progress to discern between these options and to examine the biosynthetic model in detail.

The *bta* cluster appears to provide an indication of how four analogues are biosynthesized by one cluster.[18] The hydroxyl group at C5 may be inserted by BtaA or BtaU (see above). BtaQ and BtaJ bear high identity to an acetyltransferase and an oligopeptidase, respectively. Indeed acetylated bactobolins have previously been reported.[6] *N*-Acetyl peptides are good substrates for oligopeptidases,[19] which could cleave *N*-acetyl-bactobolin B or D, generated by BtaQ, to yield bactobolin A or C, respectively. Thus, a promiscuous hydroxylase and an oligopeptidase may generate three additional congeners from bactobolin D.

Bta-based bioinformatic searches for its relatives show that *bta* is widespread in and confined to the *Burkholderia* genus (Figure 3). This suggests that the biosynthesis of OH-Cl_2-Val and its incorporation into the actinobolin framework evolved only in the *Burkholderia* lineage. The *bta* cluster is found in avirulent and virulent members of *Burkholderia*, including the important human pathogen *B. pseudomallei*.[20]

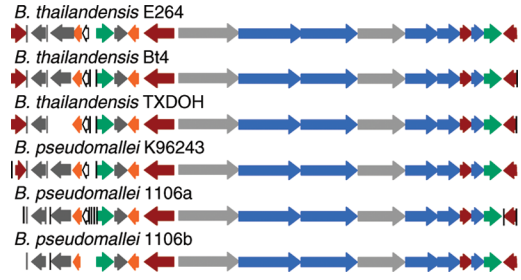

Figure 3. Results of a *bta*-based bioinformatic search using the protein database available on http://img.jgi.doe.gov. The clusters with significant homology are exclusively from sequenced *Burkholderia* strains. Only the top six results of finished sequences are shown.

(15) Secondary structure predictions show that this 300 amino acid region is SDR-like. Recent studies have suggested that another SDR-like domain involved in erythromycin biosynthesis stabilizes an enolate intermediate, which is required for enzymatic aldol reactions proposed herein. See ref 16.

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Deciphering the role of bactobolin in the quorum sensing (QS) hierarchy will be important in understanding its ecological function and the mechanism of pathogenesis within virulent *Burkholderia* strains.[3,20]

To begin to test the functional predictions above, mutational and biochemical analyses have been carried out to examine the export of bactobolin as a means of host resistance. We investigated a QS-defective mutant, which does not express *btaK-btaS* and thus does not produce bactobolin.[4] Addition of exogenous bactobolin showed that the mutant was resistant indicating that the resistance genes lie outside of this region (Tables S7 and S8 in Supporting Information).[21] BtaT is predicted to be a drug resistance transporter and may be involved in the export of bactobolin. To examine its function, an unmarked *btaT* mutation was generated (see Supporting Information). Cultivation of this mutant under the same conditions as above for 12 or 24 h showed that export of bactobolin was reduced 18-fold or 10-fold, respectively, relative to wt, supporting the role proposed for BtaT.[22] The *btaT* mutant was resistant to bactobolin, indicating additional pathways for its export, and that resistance is not dependent on *btaT* (Figure 4).[23]

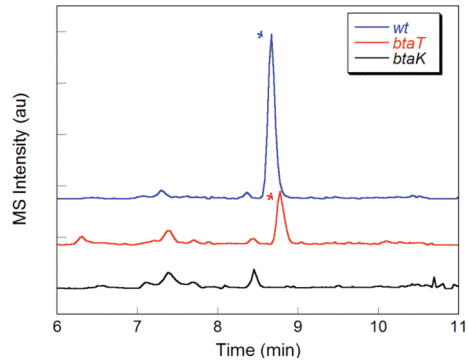

Figure 4. Ion-extracted LC-MS trace of the culture supernatants of wt *B. thailandensis* versus those of the *btaT* and *btaK* deletion mutants after 24 h of growth. The starred peaks correspond to bactobolin B. The amount analyzed for the *btaK/btaT* mutants was 3-fold that of wt. See Supporting Information for details.

To test the potency of bactobolins, antibiotic assays were carried out against the human pathogens MRSA, VRE, and *V. parahemolyticus* and against *B. subtilis* and *E. coli* (Table 1).

Table 1. Minimum Inhibitory Concentration (MIC, μg/mL)[a] of Bactobolins A, B, C, or D against Selected Bacterial Strains

strains	A	B	C	D
S. aureus COL (MRSA)	0.78	0.29	6.25	37.5
E. faecalis V583 (VRE)	>25	25	>25	nd[b]
V. parahemolyticus LM5674	0.20	0.20	1.56	nd[b]
B. subtilis 3610	1.56	0.39	12.5	100
E. coli ATCC25922	6.25	1.56	25	>100

[a] MICs were determined according to the CLSI assay (see Supporting Information). Values are the average from two independent experiments. [b] nd = not determined.

The results show that bactobolins A and B, which contain the hydroxyl group at C5, are more potent than bactobolins C and D. Bactobolin D is the weakest analogue. Bactobolins A and B are potent antibiotics against MRSA and *V. parahemolyticus* with MICs < 1 μg/mL. Bactobolin C is also effective against these strains, though to a lesser extent. All three analogues displayed low activity against VRE.

In summary, we have shown that the QS-regulated antibiotic produced by *B. thailandensis* consists of four bactobolins, of which two are potent antibiotics against human pathogens. Analysis of the *bta* cluster has allowed us to propose a biosynthetic scheme with several intriguing features: the biosynthesis of a unique OH-Cl_2-Val analogue, an unusual acyltransferase reaction to introduce OH-Cl_2-Val into the assembly line, and a set of reactions to generate an enol lactone functionality that is poorly-studied in the context of PKS reactions. The genetic accessibility of *B. thailandensis* and a plausible biosynthetic model will facilitate exploration of bactobolin biosynthesis.

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Supporting Information Available: Experimental procedures, spectroscopic data for all bactobolins and bioinformatic analysis of the *bta* cluster. This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) Exogenous bactobolin was added in the form of filtered culture supernatants of wt *B. thailandensis* E264, which contained 40 ± 18 μg of bactobolin as quantitated by LC-MS using purified standards.

(22) Bactobolin did not accumulate inside the cells of wt or Δ*btaT B. thailandensis* as determined by LC-MS methods (data not shown). The growth kinetics of these two strains were similar with doubling times of 52 ± 1 min and 51 ± 2 min, for wt and the *btaT* mutant, respectively, from two independent measurements (data not shown).

(23) The *btaK* mutant served as a control and abrogated bactobolin production as anticipated from previous bioassay data (see ref 4).