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Induced Biosynthesis of Cryptic Polyketide Metabolites in a *Burkholderia thailandensis* Quorum Sensing Mutant

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Abstract: Genetic manipulation of the LuxR-type quorum sensing regulator system in *Burkholderia thailandensis* caused a significant change in the metabolic profile: it led to activation of the thailandamide biosynthesis gene cluster, dramatically increased thailandamide production, and induced strong pigmentation. A novel polyketide metabolite, thailandamide lactone (**2**), which cannot be detected in the wild type, was isolated from the mutant broth, and its structure was elucidated by high-resolution mass spectrometry and IR and NMR spectroscopy. In a biological assay using tumor cell lines, **2** showed moderate antiproliferative activities. This finding not only points to complex regulation but also serves as a proof of concept that engineering quorum sensing mutants may enable the discovery of novel bioactive natural products encoded by silent or only weakly expressed biosynthetic pathway genes.

Microbial polyketide metabolites play a pivotal role in modern drug-based therapy of various diseases, in particular for the development of antibiotics and antitumor agents.[1] However, in recent years, it has become increasingly obvious from whole-genome sequencing projects that the biosynthetic potential of microorganisms has been greatly underexplored.[2] In many organisms, the majority of genes potentially coding for the biosynthesis of biologically active compounds remain silent, and new methods are required in order to bring such "cryptic natural products" to light.[2] It is well-conceivable that microbial cultures lack the production of metabolites under non-natural laboratory conditions, and one may consider taking specific environmental requirements of the organisms into account.[3] In many cases, variation of culture conditions, addition of chemical inducers,[3] or usage of epigenetic modifiers[4] has proved to be highly successful in activating otherwise down-regulated secondary metabolite pathways. In a more targeted approach, one may transfer and heterologously express the biosynthesis gene cluster in a suitable host. However, in view of the enormous sizes of most biosynthetic gene loci, this is often cumbersome or not yet viable. From a practical point of view, the expression of global or pathway-specific regulator genes is an attractive alternative.[5,6] Here we report a novel approach, the manipulation of quorum sensing (QS) regulation, for dramatic alteration of metabolite production. This has led to the discovery of a previously overlooked bioactive complex polyketide in the bacterium *Burkholderia thailandensis*.

B. thailandensis was isolated from rice paddies in the central and northeastern Thailand[7] and serves as a model for pathogenic relatives in the same genus (*Burkholderia mallei* and *Burkholderia pseudomallei*). Bioinformatic mining of its recently sequenced genome[8] has revealed several orphan biosynthesis gene clusters that could code for the biosynthesis of polyketide metabolites. A

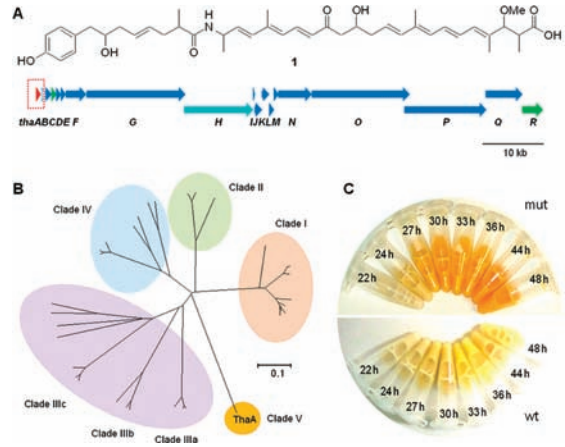

Figure 1. (A) Structure of thailandamide A (**1**) and organization of the thailandamide (*tha*) biosynthesis gene cluster, with *thaA* (LuxR gene) highlighted. (B) Phylogeny of ThaA vs LuxR homologues. (C) Strongly increased pigmentation of Δ*thaA* mutant (mut) relative to the wild type (wt).

ketosynthase (KS) analysis of a genetically encoded multimodular thiotemplate assembly line predicted an extended conjugated double-bond system and one amino acid building block.[9] On the basis of these predictions, we were able to detect the transient occurrence of two highly unstable metabolites, thailandamide A (**1**, Figure 1A) and a geometrical isomer. The metabolites are produced in only minute amounts and exclusively in the early growth phase. In order to assign the molecular basis of thailandamide biosynthesis and engineer the pathway, we investigated the putative thailandamide (*tha*) biosynthesis gene cluster. The *tha* locus consists of six large open reading frames (ORFs) coding for a multimodular NRPS/PKS hybrid (ThaGMNOPQ), a tandem acyl transferase (AT)−enoyl reductase (ER) (ThaF), a set of enzymes involved in isoprenoid-like β-branching (ThaI-M),[10] and various additional tailoring components (Figure 1A). Notably, the biosynthesis genes have a polycistronic arrangement, and the only regulatory gene found in the *tha* locus is *thaA*, which is located upstream of the predicted biosynthesis genes. The deduced 238 amino acid gene product of *thaA* shows a typical AHL-binding motif and has good overall similarity to LuxR regulators, which are known for homoserine lactone (HSL) QS.[11] In various pathogenic *Burkholderia* spp., LuxR is related to QS systems regulating virulence factors.[12] To gain insight into the function of this regulator gene, we performed a phylogenetic analysis of currently available LuxR homologues from *Burkholderia* spp. Surprisingly, the cladogram revealed that ThaA does not fall into the well-characterized clades I−IV but seems to belong to a novel group (Figure 1B). To investigate the role of this tentative, noncanonical QS regulator of the *tha* pathway, we disrupted the

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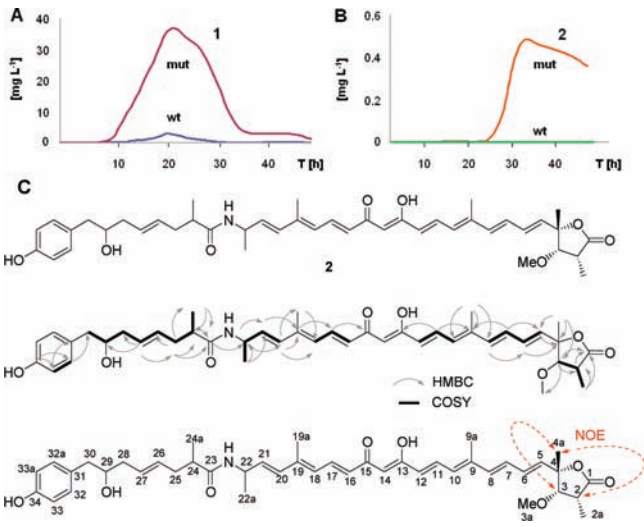

Figure 2. (A, B) HPLC monitoring of metabolite production (quantitative) in the *B. thailandensis* wild type (wt) and Δ*PthaA* QS mutant (mut): (A) **1**; (B) **2**. (C) Structure elucidation of thailandamide lactone (**2**).

ThaA coding region and the predicted transcription factor binding region through homologous recombination. We then compared the growth and metabolic profiles of the wild type and mutants. While growth was not impaired in the mutants, to our surprise, the mutant with an altered transcription factor binding region (Δ*PthaA*) accumulated a yellow pigment that was not detectable in the wild type broth (Figure 1C). Furthermore, this mutant produced thailandamide A in significantly higher amounts than the wild type (Figure 2A).

Interestingly, this yellow pigment resided within the cells and was not extractable with ethyl acetate. For isolation and full characterization, the biomass from a 20 L culture was harvested and freeze-dried. The crude yellow methanolic extract was subjected to open column chromatography, and final purification of the pigment was achieved by reversed-phase HPLC, yielding **2** and an unstable geometrical isomer. The molecular formula of **2** ($C_{43}H_{55}N_1O_8$) was deduced from high-resolution electrospray ionization mass spectrometry and ^{13}C NMR data. Through comparison of NMR data, we concluded that **1** and **2** share the same "western" fragment (C-18 to C-34) (Figure 2C). However, the "eastern" part of **2** clearly differs from that of **1**, showing various signals diagnostic of a highly unsaturated substructure. Extensive $^1H-^1H$ COSY, HSQC, and $^1H-^{13}C$ HMBC analyses eventually provided clear evidence for a polyene substructure fused through an enolized dione moiety (C-13 to C-15). Related substructures can also be found in a variety of plant-derived diarylheptanoids, such as curcumin.[13] As in these metabolites, C-13, C-14, and C-15 show characteristic weak signals due to keto−enol tautomerism. This substructure is fused to a tetraene stretch (C-5 to C-12), as evidenced by the ^3J(C,H) spin coupling of H-11 to the enol carbon (C-13, δ 184.7). The architecture of the tetraene was deduced from COSY (H-5, δ 6.04, to H-8, δ 6.48) and HMBC data. The methyl protons H-9a (δ 2.06) show a cross-peak with C-8 (δ 138.8) and C-10 (δ 131.6), and H-10 could be connected with H-11 (δ 7.66). Coupling constants and ROESY correlations indicated that the configuration of the double-bond system is all-trans. Through further 2D NMR correlations (H-4a to C-5), we found that the polyene is connected to a γ-butyrolactone ring. The carbon resonating at δ 88.4 corresponds to a quaternary alcohol (C-4) that constitutes a γ-butyrolactone ring with the C-1 carbonyl group (δ 180.1). The γ-lactone structure is supported by the diagnostic C═O band (1764 cm^{-1}) in the IR spectrum, as well as by numerous HMBC connectivities and one COSY connection. The NOESY correlations between H-4a (δ 1.50) and H-2 (δ 3.13)/H-3 (δ 3.78) revealed the relative configuration of the ring substituents of thalandamide lactone (**2**) (Figure 2C). The stable isomer **2** was subjected to bioactivity screening, which indicated moderate antiproliferative (GI_{50} = 28 μM) and cytotoxic (CC_{50} = 32 μM) activities on HUVEC and HeLa cells, respectively.

It appears that the *tha* biosynthesis gene cluster is silent or significantly down-regulated under regular growth conditions. To monitor *tha* PKS gene expression, we compared expression profiles for the wild type and mutant strains by quantitative RT-PCR. Our data clearly demonstrate that PKS gene expression is only basal and only detectable during a short time window in the wild type. In the Δ*PthaA* QS mutant, however, PKS gene expression is substantially enhanced over an extended period of time, while the loss of *thaA* abolishes polyketide biosynthesis. In most antibiotic biosyntheses, LuxR homologues function as positive regulators, as in the carbapenem,[14] fosfomycin,[15] pyrrolnitrin,[16] pimaricin,[17] and geldanamycin[18] pathways. It has also been reported that a *B. thailandensis* mutant in which another *luxR* copy was deleted lost the ability to produce the antibiotic bactobolin.[19] Interestingly, various studies have shown that LuxR QS is often oversimplified, and several cases in which LuxR homologues are involved in negative regulation are known.[20] Our results imply that thailandamide production is controlled by a complex regulatory system involving ThaA-mediated QS that seems to be repressed under standard cultivation conditions. To our knowledge, the up-regulation of a cryptic biosynthetic pathway through disruption of a QS regulator component is unprecedented.

In summary, we have activated the *tha* PKS-NRPS gene cluster in *B. thailandensis* through manipulation of a QS regulatory system involving a gene coding for a LuxR homologue. In addition, we have unequivocally proved the identity of the *tha* biosynthesis gene cluster through gene inactivation and RT-PCR monitoring of increased PKS gene expression in the engineered strain. Through genetic manipulation of the operon, we were able to alter dramatically the metabolic profile of the mutant and isolate a novel, structurally intriguing polyketide named thailandamide lactone (**2**). In a biological assay using tumor cell lines, **2** showed moderate antiproliferative activities. This is a proof of concept that engineering QS regulation may enable the discovery of novel bioactive natural products. The approach holds promise to be generally applicable to activation of silent or weakly expressed pathway genes that are under control of LuxR homologues in other bacteria.

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Supporting Information Available: Experimental procedures, HPLC profiles, growth curves, RT-PCR data, phylogeny, and 1D and 2D NMR spectra of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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