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Genomics-Driven Discovery of Burkholderic Acid, a Noncanonical, Cryptic Polyketide from Human Pathogenic *Burkholderia* Species**

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Bacteria belonging to the genus *Burkholderia* comprise a multifarious group of microorganisms that play eminent roles in highly diverse ecological niches.[1] However, *Burkholderia* spp. are perhaps best known for the human and animal pathogens, *Burkholderia mallei* and *Burkholderia pseudomallei*. *B. mallei* is the causative agent of glanders, an infamous disease primarily affecting horses and related ungulates.[2] Nonetheless, the pathogen may be transferred to humans, and shockingly high mortality rates (95% without treatment, 50% with treatment) have been reported. *B. pseudomallei* is known as the causative agent of melioidosis, a human infectious disease that is endemic in Southeast Asia and Australia with mortality rates between 20 and 50%, even with treatment in highly developed areas.[3,4] In this context it is noteworthy that these severe pathogens can enter a dormant state after infection, after which they may reactivate and cause relapse or the onset of acute melioidosis.[3,4] Unfortunately the molecular basis of virulence and the triggers and mechanisms underlying the diverse clinical manifestations of the diseases are only poorly understood.[5,6] Overall, surprisingly little is known about the biosynthetic potential and small-molecule virulence factors of *Burkholderia* spp.[7] This lack of knowledge is particularly remarkable since full genome sequencing of the pathogens[8,9] has unveiled a number of gene clusters that code for secondary metabolite pathways. However, biosynthetic and metabolic studies in this area are challenging, because *B. mallei* and *B. pseudomallei* are considered potential biological warfare agents.[6] Phylogenetically related albeit less virulent *Burkholderia thailandensis* strains are thus commonly used as model organisms for pathogenesis.[10] Herein we report the discovery of a highly unusual, cryptic polyketide pathway that is conserved in all bacteria belonging to the *B. pseudomallei* group. Through the targeted activation of the silent gene cluster, combined with chemical and bioinformatic analyses, we not only unveil the architecture of a structurally intriguing polyketide metabolite, but also shed first light on an unprecedented assembly line generating a furan derivative from two polyketide chains.

When comparing the genomes of the related pathogens *B. mallei*, *B. pseudomallei*, and *B. thailandensis*, we noted that these three organisms share an enigmatic gene locus (*bur*) that codes for a modular assembly line composed of unusual polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) modules (Figure 1 A). In light of the importance and thorough investigation of these pathogens it is remarkable that to date no metabolites have been assigned to the tentative biosynthetic gene clusters. To get an idea of the nature of the potential metabolite encoded, we performed bioinformatic analyses of the *bur* biosynthetic locus. Typically,

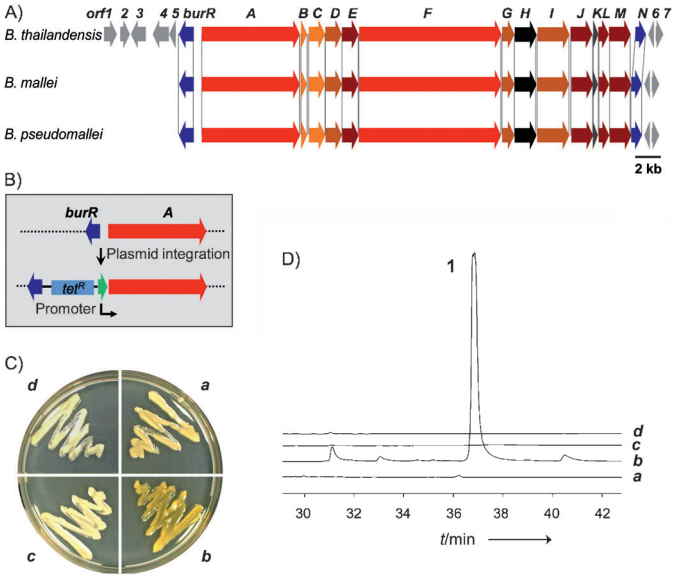

Figure 1. Architecture and targeted activation of related cryptic PKS gene clusters distributed among pathogenic *Burkholderia* spp. A) Comparison of the *bur* gene clusters in *B. thailandensis*, *B. mallei*, and *B. pseudomallei*. Deduced functions: BurR, LuxR-type regulator; BurA, NRPS/PKS; BurB, methyltransferase; BurC, hydroxylase; BurD, aminotransferase; BurE, dehydrogenase; BurF, NRPS/PKS; BurG, ketol acid reductoisomerase; BurH, FkbH-like protein; BurI, decarboxylase; BurJ, acyl CoA ligase; BurK, unknown; BurL, acyltransferase; BurM, acyl CoA ligase; BurN, efflux carrier protein. B) Promoter replacement for triggering *bur* gene expression. C) Phenotypes of *B. thailandensis* wild type (a), *Pbur* mutant (b), *Pbur*Δ*burA* mutant (c), *Pbur*Δ*burF* mutant (d). D) HPLC profiles of extracts from liquid cultures of wild type and mutants; diode-array detection (DAD), $\lambda = 374$ nm.

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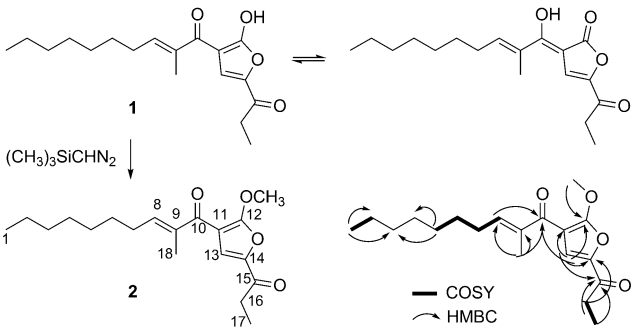
modular PKS constitute linear thiotemplate systems,[11,12] and many impressive studies have demonstrated that it is possible to envisage the backbone of the final product from the encoded assembly line.[13] However, the noncanonical architecture of the deduced PKS and NRPS modules is intriguingly enigmatic, thus hampering a sound structure prediction (Figure 3). Not only are PKS and NRPS modules incomplete or disrupted, two thioesterase (TE) domains are distributed within the modules at highly unusual loci. Furthermore, the *bur* cluster codes for various accessory genes, such as an acyltransferase (AT), BurL, and a dehydratase (DH), BurE, which may serve the PKS in trans (that is, not as part of the module).[14] Through expression analyses we found that *bur* pathway is silent under standard growth conditions (data not shown). Obviously, like many microbial biosynthetic gene clusters the *bur* genes are downregulated and only activated in the presence of particular, yet unknown stimuli.[15,16] After analyzing the components of the gene locus we reasoned that the gene cluster could be under control of the tentative LuxR-type regulator BurR, which is encoded upstream of the NRPS–PKS gene *burA*. However, overexpression and deletion of *burR* did not result in any obvious change in the metabolic profiles. Since the *bur* locus has a polycistronic arrangement, we next envisaged the possibility of gene cluster activation by promoter exchange, using techniques and tools established for analyzing the thailandamide gene cluster in *B. thailandensis*.[17–19] Through insertional replacement we introduced a cassette including a constitutive promoter and a tetracycline resistance gene upstream of *burA* (Figure 1 B). Interestingly, the mutant exhibited a clear change in phenotype, showing strong yellow pigmentation. HPLC–HRMS monitoring of the mutant broth revealed that the promoter exchange triggered the formation of a new compound (**1**) with m/z $[M-H]^- = 305.1759$, which corresponds to a molecular formula of $C_{18}H_{26}O_4$. Surprisingly, the isolation and structure elucidation of the yellow pigment proved to be extremely challenging. Initial isolation attempts indicated that compound **1** is highly unstable, since pure samples readily degraded within a few hours. Although we thoroughly avoided elevated temperatures and exposure to light and oxygen, it was impossible to get hold of a pure sample. To circumvent these problems, we developed an optimized, rapid isolation process involving subsequent chloroform extraction and purification by preparative HPLC. Although we succeeded in isolating pure **1** in good yields (2–30 mg L^{-1}, depending on culture conditions and fitness of strains) within less than five hours, the inherent instability of the new compound prevented recording 2D NMR spectra in sufficient quality. Since we realized that severe oxygen sensitivity of compound **1** caused the problem, we added the potent radical scavenger BHT (butylated hydroxytoluene) to the sample to slow down the decay. This procedure eventually allowed measurement of a complete 2D NMR data set. Whereas substructures of **1** could already be deduced from these data, a noisy background owing to beginning decomposition hampered a rigorous allocation of the full set of carbon signals. Furthermore, it appeared that compound **1** may exist in different tautomeric forms. To unequivocally assign the structure of **1** we thus sought to prepare a stable derivative. Since compound **1** showed typical behavior of an acidic compound during chromatography and mass spectrometry, we decided to generate the methyl derivative. Indeed trimethylsilyldiazomethane-mediated methylation yielded a stable derivate **2** (Scheme 1), which showed the expected



Scheme 1. Structures of burkholderic acid (**1**), the tautomer and methylated derivative **2**; key COSY and HMBC correlations.

mass shift in the MS spectrum of 14 Da that accounts for an additional methyl group. NMR spectroscopy measurements of **2** were in good agreement with the data obtained for compound **1**, thus indicating that the core structure remained unaffected. For the methylated derivative **2** a molecular formula of $C_{19}H_{28}O_4$ was deduced from its HRMS and ^{13}C NMR data. The DEPT135 spectrum indicated the presence of four methyl groups, one of which was introduced by methylation ($\delta_C = 59.0$ ppm), seven methylene groups, two methine groups, and six quaternary carbon atoms. A propionyl side chain could be readily identified by an ethyl spin system and correlations to $\delta_C = 190.2$ ppm. According to COSY and HMBC correlations the remaining six methylene groups constitute a heptyl side chain attached to a trisubstituted double bond. The chemical shifts of the olefinic carbon atoms of $\delta_C = 145.4$ ppm and 137.3 ppm indicated an α,β-unsaturated system. HMBC correlations to a quaternary carbon with $\delta_C = 192.5$ ppm fully supported this finding. A methyl group was revealed as the third substituent by HMBC correlations and a $^4J_{HH}$ coupling of 1.0 Hz. Its chemical shift of $\delta_C = 12.2$ ppm indicated a *trans* configuration of the double bond. To complete the structure, four carbon atoms with $\delta_C =$ 103.0 ppm, 123.7 ppm, 143.2 ppm, and 164.4 ppm and two oxygen atoms had to be placed, accounting for an acidic functionality and three double bond equivalents. Therefore, **2** had to be monocyclic. The methylated carbon atom ($\delta_C =$ 164.4 ppm) was unambiguously identified based on HMBC correlations, which defined the positions for the remaining oxygen atoms. Owing to the low-field shift of C-14 ($\delta_C =$ 143.2 ppm) it was obvious that the cyclic system represents a trisubstituted furan derivative. This result is fully supported by the chemical shift of the ring proton ($\delta_H = 7.55$ ppm) and the detection of all expected ^2J and ^3J HMBC correlations. Thus, **2** features a 2-methoxyfuran core, whereas **1** is a 2-hydroxyfuran derivative, which accounts for the acidity. It should be noted that 2-hydroxyfurans tautomerize into butenolides; this tautomerization leads to an enlarged

conjugated system in the case of **1**. However, methylation locked the compound in its hydroxyfuran form as evidenced by the shift of λ_{max} from 374 nm for **1** to 305 nm for **2**, which accordingly was colorless. Further support for the tautomery was provided by the downfield shift of C-15 $\delta_C = 176.7$ ppm for **1** to 190.2 ppm for **2**.

In this way we fully elucidated the structures of the yellow pigment **1**, named burkholderic acid because of its origin and acidity, and of its stable derivative **2**, methyl burkholderate. In a preliminary screening for antimicrobial and cytotoxic properties, **1** and **2** showed no antibacterial potencies and only weak to moderate cytotoxicity. Since the substituted hydroxyfuran and tautomeric butenolide have marked structural similarities with quorum sensing molecules such as A-factor and *N*-acyl homoserinelactones (AHL),[20] the short-lived molecule may be involved in microbial communication processes. This role is likely given the quorum sensing regulator (*luxR*-like) gene that sits upstream of the cluster. Future in vivo infection experiments will clarify whether **1** contributes to the virulence of the pathogenic *Burkholderia* spp.

On biosynthetic grounds, the structure of **1** is highly intriguing since the disubstituted hydroxyfuran is likely constituted by a branched polyketide backbone. Actually, the architecture of **1** is quite unexpected when it is taken into account that the deduced gene products of *burA* and *burF* feature noncanonical PKS as well as NRPS-like modules or domains, thereby suggesting that at least one amino acid would be incorporated into the corresponding metabolite. To firmly correlate the biosynthesis of **1** with the *bur* PKS genes, we deleted these two genes in the overexpression mutant by replacement with a kanamycin resistance cassette. Production of the yellow pigment was completely abrogated in plate cultures of both mutants, *B. thailandensis* E264 *Pbur*Δ*burA* and *Pbur*Δ*burF* (Figure 1 C). The shutdown of burkholderic acid production was further confirmed by RP-HPLC-MS monitoring of EtOAc extracts from liquid cultures of the mutants, thereby clearly linking BurA and BurF to the biosynthesis of **1**.

To gain first insights into the biosynthesis of the unusual polyketide backbone we performed stable isotope labeling experiments. After addition of 1-^{13}C-acetate and 1,2-$^{13}C_2$-acetate to the culture, we noted substantially increased signal intensities in the ^{13}C NMR spectra of labeled **1**. From these data we inferred that the entire carbon skeleton except for the terminal propionyl residue is composed of acetate units. Although position and substitution of the residual C_3 unit would suggest that this moiety is derived from propionate, quite unexpectedly, ^{13}C-labeled propionate was not incorporated into **1**. We thus reasoned that the propionyl moiety could originate from an amino acid, which may be converted by the putative aminotransferase BurD and decarboxylase BurI. To test this hypothesis, we administered various labeled amino acids with appropriate carbon skeletons including aspartate and glutamate, yet none of these were incorporated into **1**. Searching for alternatives we noted that a tentative D-methionine ABC transporter is encoded upstream of the *bur* gene cluster. Indeed, addition of [D_4]-3,3,4,4-methionine lead to a pronounced mass shift of **1** by 4 Da (Figure 2). This

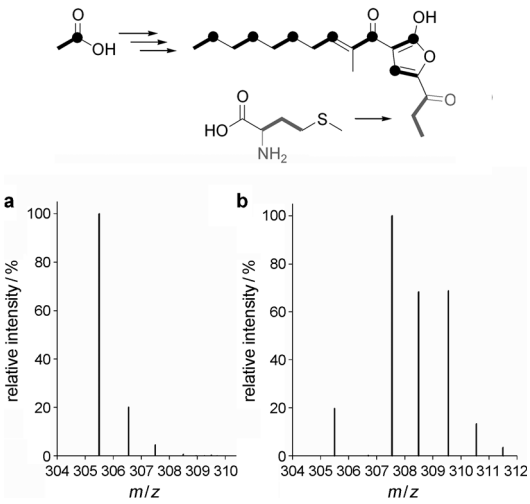


Figure 2. Results from stable isotope labeling experiments. The bolded black bonds indicate which parts of the skeleton originate from acetate. Mass spectra of native **1** (a) and **1** labeled with [D_4]-3,3,4,4-methionine (b). (The occurrence of M + 2 and M + 3 shifts can be rationalized by deuterium leaking in the α-position.)

finding is quite surprising, because to our knowledge the incorporation of a methionine-derived C_3 unit into a polyketide backbone is completely unprecedented.

From the genetic and chemical data we developed a plausible biosynthetic model that involves the fusion of two polyketide chains (Figure 3 A). The longer chain is built from a short-chain fatty acid that is activated by the CoA ligase BurJ. This uncommon octanoate PKS starter unit[21] is loaded onto the N-terminal ACP of BurF and elongated by two PKS modules in a near colinear fashion. In parallel, the shorter chain would be initiated by a propionyl unit derived from methionine by transamination, decarboxylation, and desulfurization in analogy to the cystathionine γ-lyase reaction.[22]

Next, the structure of **1** suggests that propionyl ACP is formally elongated with a hydroxymalonyl unit.[23] However, the AT domain shows specificity for malonyl CoA, and thus we reason that either malonyl ACP or the downstream product undergoes α-hydroxylation. The best candidate for this reaction is BurC, a putative hydroxylase with high similarity to the hydroxylase SyrP from the syringomycin (*syr*) pathway. Notably, in vitro studies showed that SyrP acts on Asp tethered to a thiolation domain on the *syr* NRPS.[24] Furthermore, the model involving chain hydroxylation is well-supported by labeling studies, which fully rule out the typical hydroxymalonyl-ACP pathway originating from glycolysis intermediates.[23] Finally, both polyketide chains are linked through an ester bond; this esterification is most likely catalyzed by the condensation (C) domain of BurF. Indeed, there is a compelling precedent for ester formation by a freestanding C domain in the biosynthesis of the enediyne C-1027,[25] and ester formation would also be the most plausible scenario in burkholderic acid biosynthesis. The final ring formation is reminiscent of tetramic acid[26] and tetronate[27] biosynthesis. Indeed, we noted a reductase domain at the C terminus of BurF; this finding indicates

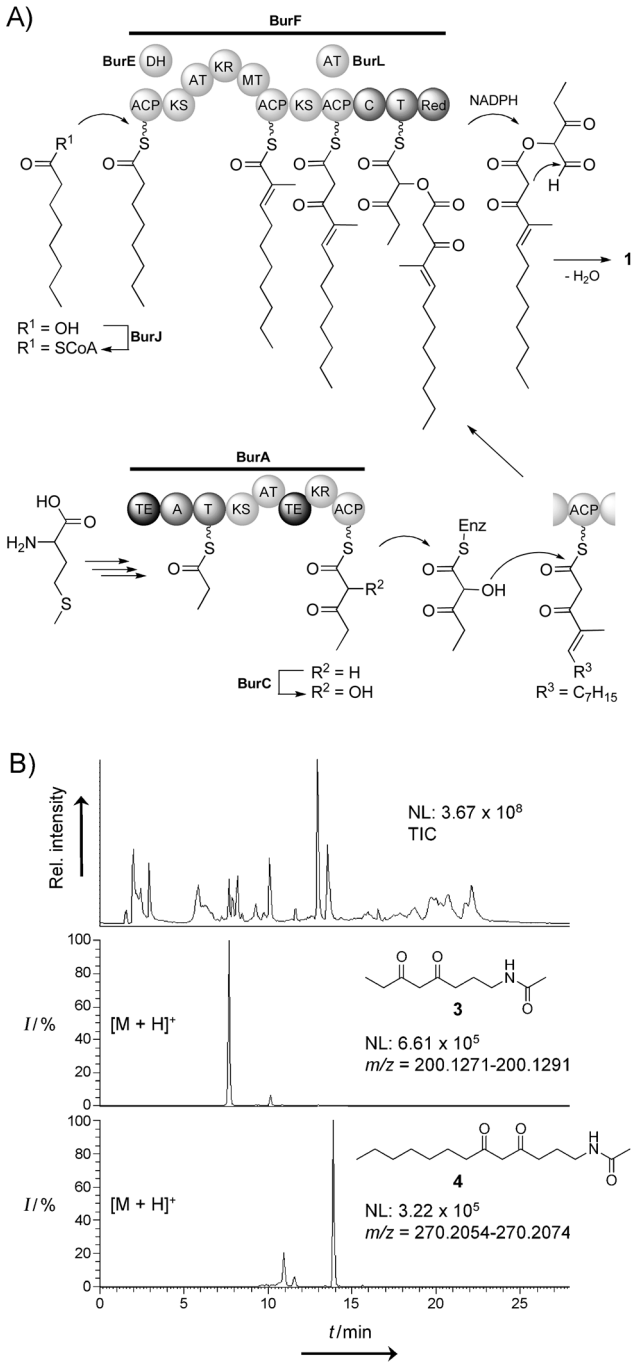


Figure 3. A) Model for burkholderic acid biosynthesis. CoA, coenzyme A; KS, ketosynthase; AT, acyltransferase, KR, ketroreductase; DH, dehydratase, MT, methyltransferase; ACP, acyl carrier protein; C, condensation; T, thiolation, Red, reductase; TE, thioesterase; A, adenylation, Enz, enzyme. B) Detection of pathway intermediates from both PKS using synthetic malonyl-CoA mimics as chain terminators. NL = neutral loss, TIC = total ion count.

a reductive chain release in analogy to fungal PKS–NRPS involved in cyclopiazonic acid and cytochalasin biosynthesis.[28,29] From a chemical point of view a liberated aldehyde could readily undergo a Knoevenagel-type cyclocondensation.

To corroborate the biosynthetic model involving two independent polyketide chains involved in burkholderic acid biosynthesis, we engineered a mutant PKS lacking the TE domain in BurF. Similarly mutated *trans*-AT PKSs impaired in off-loading have released immature polyketide precursors[30] owing to the proof-reading of an AT-like enzyme.[31] However, since no intermediates could be detected, it seems that such an activity is not available for the *bur* PKS. Thus, we tested mimics of malonyl-CoA surrogates as synthetic chain terminators, which have been shown to off-load intermediates from a PKS.[32] Indeed, masses for the predicted pathway intermediates of both artificially terminated polyketide chains, **3** and **4**, could be detected by LC–HRMS analyses (Figure 3B).

In summary, we have discovered a cryptic NRPS–PKS gene cluster that seems to be a hallmark of well-studied pathogenic bacteria belonging to the *B. mallei* group; yet the corresponding metabolites have been overlooked to date. Through a directed promoter replacement we succeeded in activating the silent pathway in *B. thailandensis*. However, the isolation and structure elucidation of the highly oxygen-sensitive metabolite proved to be particularly challenging. We achieved this goal by using an optimized, rapid purification protocol in the presence of a radical scavenger. Furthermore, a stable methylated derivative unequivocally corroborated the highly unusual polyketide scaffold of burkholderic acid. Through gene deletions the involvement of noncanonical PKS–NRPS modules in the biosynthesis of **1** was proven. Stable isotope labeling experiments and domain analyses have not only revealed that the C_3 unit is—most surprisingly—derived from methionine, but have also shown a new example of the head-to-head fusion of two polyketide chains. In contrast to the corallopyronin[33] and the *Xenorhabdus* stilbene pathways,[34] which also involve mergers of two polyketide chains, the fusion of the burkholderic acid subunits does not involve a KS but seems to be unique in utilizing a C domain. According to a plausible biosynthetic scheme the rare furan chromophore results from reductive chain release with concomitant Knoevenagel-type cyclocondensation. We believe that the discovery of burkholderic acid and the characterization of the *bur* gene cluster are significant, because the locus is conserved in the infamous *B. mallei* and *B. pseudomallei* pathogens, for which no polyketide metabolites have been described to date. The structure and short lifetime of **1** suggest that it may be involved in signaling processes, possibly contributing to the virulence of the causative agents of melioidosis and glanders. Notwithstanding, burkholderic acid is a fresh addition to the very few (nonsiderophore) PKS and NRPS products known from human pathogenic bacteria.[35] Thus, our study not only provides unprecedented metabolic data for a cryptic gene cluster that is conserved in human pathogens, but also provides first insights into a fascinating polyketide pathway featuring novelties regarding building block biosynthesis, chain fusion, and furan formation.

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