

**Global awakening of cryptic biosynthetic gene clusters  
in *Burkholderia thailandensis***

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## Abstract

Many bacteria encode biosynthetic proteins that produce a vast array of natural products. These compounds are often synthesized during host invasion as they function as virulence factors. In addition, such secondary metabolites have yielded numerous molecular scaffolds with pharmaceutical and clinical importance. The gene clusters that encode proteins responsible for synthesis of these compounds are typically silenced or “cryptic” under laboratory growth conditions, hampering discovery of novel lead compounds. We report here that MftR is a global repressor of secondary metabolite synthesis in *Burkholderia thailandensis* and that urate functions as a physiologically relevant inducer of gene expression. Biosynthetic gene clusters under MftR control include those associated with production of the antimicrobial bactobolins, the iron siderophore malleobactin, and the virulence factor malleilactone. MftR also controls additional genes associated with survival in a host environment, such as genes encoding components of the type III secretion system (T3SS) and proteins linked to anaerobic respiration. This observation not only has implications for understanding activation of gene regulatory networks during host invasion, but it also paves the way for isolation of novel therapeutic leads.

## Introduction

The ecologically diverse genus *Burkholderia* includes the serious human pathogens *B. pseudomallei* and *B. mallei* and the closely related *B. cepacia* complex opportunistic pathogens, which primarily infect immunocompromised individuals such as persons with cystic fibrosis.<sup>1-3</sup> *B. thailandensis* is a saprophyte that only causes mammalian infections at high doses. However, it conserves a number of genes encoding factors that are critical for pathogenicity of the more virulent species, such as the type III secretion system and quorum sensing systems that are key to control of virulence gene expression.<sup>4</sup>

*B. thailandensis* also shares with the virulent species a number of gene clusters that include predicted polyketide synthase (PKS) or nonribosomal peptide synthetase (NRPS) genes that are associated with synthesis of natural products. Such compounds include iron siderophores and toxins that enable the bacteria to compete with other species and they are known to confer increased fitness and to play important roles in establishment and propagation of infection.<sup>5, 6</sup> Biosynthetic gene clusters are usually tightly regulated, resulting in silencing or low-level expression under standard growth conditions, and identification of inducers of gene expression *in vivo* has proven challenging. While the structures of several natural products have been elucidated and their biological function deduced, the products of several such gene clusters remain to be identified. Since many clinically relevant antibiotics and other therapeutics are derived from natural products, triggering the expression of these cryptic gene clusters under laboratory growth conditions is of significant interest.<sup>7, 8</sup>

Quorum sensing (QS) may contribute to awakening of these silent gene clusters. This cell-cell signaling process is designed to sense population density and it involves the

synthesis and secretion of signaling molecules that are detected by other members of the population, thereby allowing the bacteria to synchronize their gene expression programs.<sup>9</sup>

<sup>10</sup> Such signaling molecules include N-acyl-homoserine lactones (AHLs), which are synthesized by members of the LuxI protein family and detected by LuxR-type regulators. Gene regulation by LuxR typically requires binding of the cognate AHL. In *B. thailandensis*, QS has been implicated in controlling expression of ScmR, a LysR-family transcription factor that in turn regulates expression of several biosynthetic gene clusters.<sup>11</sup>

*B. thailandensis* and *B. pseudomallei* encode three AHL-based QS systems, of which two are conserved in *B. mallei*. In *B. thailandensis*, BtaI1-BtaR1 signals through a homoserine lactone (HSL) containing an 8-carbon acyl chain (C<sub>8</sub>-HSL), BtaI2-BtaR2 (which is not conserved in *B. mallei*) signals through a hydroxylated AHL (3OHC<sub>10</sub>-HSL), and BtaI3-BtaR3 signals through 3OHC<sub>8</sub>-HSL <sup>12-14</sup>. In addition, two orphan LuxR homologs that lack a cognate LuxI have been identified in *B. thailandensis*, BtaR4 (also named MalR) and BtaR5.

The QS systems are interconnected and linked to signaling through a separate class of compounds. Originally characterized in *Pseudomonas aeruginosa*, 4-hydroxy-2-alkylquinolines (HAQs) may have antimicrobial activity and may chelate iron, but some also contribute as signaling molecules to control expression of virulence genes.<sup>15-17</sup> The operon encoding 4-hydroxy-2-heptylquinoline (HHQ) is conserved in *B. thailandensis* (and *B. pseudomallei* and *B. cenocepacia*) and extended by two additional open reading frames to generate *hmqABCDEFG*, responsible for synthesis of the methylated HHQ derivative 4-hydroxy-3-methyl-2-alkylquinoline (HMAQ).<sup>18, 19</sup> It has been suggested that only the methylated derivatives play a role in quorum sensing in *Burkholderia* spp. since HHQ does not appear to accumulate.



We have previously characterized the transcription factor MftR from *B. thailandensis* and shown that repression of the *mftR* gene and a divergent operon is relieved by urate.<sup>20, 21</sup> Urate is the product of xanthine dehydrogenase (XDH), which is also a key enzyme in purine salvage (Fig. 1C). In mammalian cells, bacterial infection may lead to conversion of XDH to xanthine oxidase, which produces reactive oxygen species (ROS) in addition to urate as an initial defense against the infection.<sup>22, 23</sup> Bacterial infections have been reported to result in urate levels in excess of 200  $\mu$ M and to be worsened by enhanced xanthine oxidase activity.<sup>24</sup> This suggests that bacterial pathogens may encounter urate due to activation of host xanthine oxidase and that urate may function as a signaling molecule to elicit virulence gene expression.

We show here that MftR functions as a global repressor of numerous biosynthetic gene clusters and quorum sensing systems and as a regulator of other genes linked to survival in a host environment. This observation not only has implications for understanding activation of gene regulation networks during the environmental stress encountered during host invasion, but it also paves the way for isolation of novel therapeutic scaffolds by upregulating cryptic natural product gene clusters.

## Results and Discussion

While several bacterial species encode transcription factors that share homology with MftR, including the ability to bind urate,<sup>25-27</sup> MftR is mainly encoded by *Burkholderia* species (Fig. S1). Members of the *B. pseudomallei* group that includes *B. thailandensis* conserve the *mftP-fenI* operon encoding a predicted efflux pump and a FenI family enzyme with predicted glycosyl hydrolase activity (Fig. 1A), whereas *B. cepacia* complex pathogens

lack *fenI*. In contrast, phytopathogens and environmental species such as *B. xenovorans* and *B. glumae* encode an MftR homolog in a different genomic context. These distinctions closely match the recent phylogenetic classification of *Burkholderia* spp.<sup>3</sup> To confirm the predicted *mftP-fenI* operon, cDNA was prepared using a *fenI*-specific primer, followed by PCR amplification using *mftP*-specific primers. A product of the expected size was obtained, confirming the annotated operon (Fig. 1B).

**The MftR regulon.** Repression of the predicted *mftP-fenI* operon by MftR was verified by targeted deletion of *mftR* followed by determination of transcript levels. As shown in Fig. 2A, expression of *mftP* was increased ~80-fold in *mftRΔ*, while complementation with plasmid-encoded MftR completely restored repression. Consistent with observed repression, confirmed binding sites for MftR overlap with the predicted promoter elements for the divergent genes (Fig. S2A-B).

Transposon-insertion in *mftP* (expected to be polar and to disrupt expression of *fenI* as well) resulted in very marked clumping during growth (Fig. 2C). Complementation with *mftP* did not prevent clumping, rather it resulted in the clumps attaining an orange tint, and expression of *mftP-fenI* was required to restore the wild-type growth phenotype. This indicates that absence of FenI is associated with clumping and conversely, that the increased expression of *fenI* that occurs in presence of the inducing MftR ligand (urate) would be expected to promote dispersal of cells. *Burkholderia* species produce several exopolysaccharides (EPS), but mainly cepacian, a major virulence determinant and participant in surface adhesion and biofilm maturation.<sup>28</sup> FenI-mediated cleavage of glycosidic linkages between constituent sugars in EPS would be consistent with reduced

clumping; a similar inhibition of biofilm formation as a result of EPS cleavage by the glycoside hydrolase dispersin B was previously reported.<sup>29</sup>

To define the MftR regulon, we isolated total RNA from wild-type *B. thailandensis* E264 and the corresponding *mftRΔ* strain after culturing each strain in absence or presence of 5 mM urate. The cDNA reads were mapped to the corresponding genes on the two *B. thailandensis* chromosomes. Comparison of genes exhibiting a difference in expression level of at least two-fold ( $q < 0.01$ ) in wild-type and *mftRΔ* strains yielded 331 genes upregulated in absence of MftR (Table S1) and 70 genes downregulated (Table S2), indicating that MftR functions as a repressor in most instances. A comparison of genes differentially expressed on addition of urate to the wild-type strain yielded 321 genes upregulated (Table S1) and 45 genes downregulated at least two-fold in presence of urate (Table S2).

Examination of functional categories of genes that are differentially expressed on deletion of MftR or on addition of urate to WT cells revealed significant overlap (Fig. 3). In general, genes associated with pathogenesis are downregulated in *mftRΔ* or in presence of urate, whereas genes linked to metabolic or biosynthetic processes or transport are upregulated. In addition, genes encoding MftR, MftP, and FenI were significantly upregulated (10-30-fold) in *mftRΔ*, as expected. Notably, urate did not result in upregulation of these genes, which were previously seen to be upregulated ~14-fold when cultures were grown with 10 mM urate.<sup>21</sup> The affinity of MftR for each of the two DNA sites in the *mftR-mftP* intergenic DNA is ~0.7 nM and its affinity for urate is ~6 μM.<sup>21</sup> The Cheng-Prusoff equation<sup>30</sup> relates the IC<sub>50</sub> (the concentration of inhibitor – urate in this case – required for half-maximal inhibition) and affinity for the ligand that is being competed off

(DNA). When the affinity for DNA is very high, the Cheng-Prusoff equation predicts that  $IC_{50}$  will be greater than the inhibition constant  $K_i$  (which corresponds to the  $\sim 6 \mu M$  affinity for urate), whereas the  $IC_{50}$  would be closer to the  $K_i$  when the affinity for DNA is low. We therefore considered if induction of *mftP* expression would be dependent on the concentration of urate. As shown in Fig. 2B, *mftP* expression was not induced at 1 mM urate and it increased with urate concentration, with  $\sim 7$ -fold induction with 8 mM urate. Combined with the poor solubility of urate, which confounds an estimate of actual concentrations in the culture, we infer that higher urate levels are required to relieve MftR-mediated repression of *mftR* and *mftP-fenI*. This observation also leads to the prediction that gene promoters to which MftR binds with lower affinity may be more sensitive to urate. The physiological implication is that some genes are induced at a urate concentration at which others remain repressed, perhaps effecting a temporal pattern of gene induction as the MftR ligand accumulates.

**Differential expression of genes associated with anaerobic growth.** Deletion of *mftR* or addition of urate to WT cells led to 5-10-fold upregulation of genes encoding proteins that participate in the arginine deiminase pathway (*arcDABC*; *BTH\_I2383-2386*; Table S1). This pathway has been implicated in anaerobic growth on rich medium, with the final step generating ATP.<sup>31-33</sup> Also upregulated were divergent operons encoding proteins required for synthesis of poly-hydroxybutyrate (PHB; *BTH\_II0416-0418*) and ATP synthase (*BTH\_II0419-0427*), respectively. ATP synthase utilizes the proton gradient for ATP synthesis, but it also functions to hydrolyze ATP to increase proton-motive force, a function that has been linked to survival under anaerobic conditions in *P. aeruginosa*.<sup>32</sup> Upregulation of *atpA-2* encoding F1F0 ATP synthase  $\alpha$  subunit on deletion of MftR or on addition of 5

mM urate to WT cells was verified by qRT-PCR (Fig. S3). PHB and other polymers are synthesized as carbon and energy reserves, and they are of commercial interest as biodegradable plastics.<sup>34</sup> In oxygen-deprived *B. pseudomallei*, several classes of genes were upregulated, including *arcDABC*, ATP synthase genes, genes encoding PHB, and general stress-response genes.<sup>35</sup> Homologs of the latter in *B. thailandensis* include *BTH\_I11569* and *BTH\_I12321*, which are modestly upregulated in *mftRΔ* (~two-fold). Evidently, deletion of MftR resulted in upregulation of several genes that are also induced under anaerobiosis. Consistent with elevated expression of such genes, *mftRΔ* cells exhibited greater survival when maintained under low oxygen conditions for ~5 h, following which survival of *mftRΔ* cells approached that of WT cells (Fig. 4A).

During anaerobic growth, nitrate is the favored electron acceptor, and respiratory nitrate reduction is much more efficient compared to the arginine deiminase pathway. However, concurrent signals of low oxygen and presence of nitrate (or a metabolite thereof) is required for expression of respiratory nitrate reductase genes. These genes are activated by NarL, which is part of the NarXL two-component system that responds to nitrate. In *P. aeruginosa*, NarL was also reported to repress the *arcDABC* operon.<sup>36</sup> Notably, *B. thailandensis narXL* genes (*BTH\_I1849-1850*) were downregulated ~6-fold in *mftRΔ*. It is therefore conceivable that reduced expression of *B. thailandensis* NarL is responsible for the observed increase in *arcDABC* expression.

The expression of nitrate reductase genes (*BTH\_I1851-1856*) was also reduced in *mftRΔ*, consistent with reduced expression of the activator NarL. While *narXL* genes were also downregulated on addition of urate, nitrate reductase genes were upregulated (Table S1), suggesting a urate-mediated regulation that does not depend on MftR. One of the end-

products of urate metabolism is ammonia; likewise, an increase in arginine metabolism as inferred from upregulation of *arcDABC* would also lead to accumulation of ammonia. Considering that several *Pseudomonas* species, which are closely related to *Burkholderia*, have been reported to exhibit heterotrophic nitrification activity,<sup>37</sup> it is possible that *B. thailandensis* is likewise capable of converting ammonia to nitrite and nitrate; accumulation of these compounds would function with NarXL to upregulate cognate genes. That *narXL* expression is reduced in presence of urate may serve to balance expression of the respiratory nitrate reduction pathway with that of arginine fermentation.

**Repression of genes encoding T3SS components in *mftRA*.** The type III secretion system (T3SS), which functions to deliver effector toxins into host cells is critical for virulence.<sup>38</sup> The *B. pseudomallei* T3SS is required for escape from the phagosome into the host cytosol, and it is downregulated during growth in macrophages.<sup>39, 40</sup> A regulatory cascade has been reported in which BsaN is required for activation of genes encoding T3SS effector and translocon components.<sup>41</sup> In *B. thailandensis*, expression of genes encoding the T3SS was reduced in *mftRA* and on addition of urate (that would be produced by cytosolic xanthine oxidase), perhaps reflecting its role only during early stages of infection. The *B. thailandensis* *bsaN* gene is among those downregulated (~6-fold), which could explain the similarly reduced expression of other T3SS genes. The downregulation of *bsaN* in *mftRA* or on addition of urate to WT cells was verified by qRT-PCR (Fig. S4).

**Regulation of biosynthetic gene clusters and quorum sensing.** For enumeration of differentially expressed biosynthetic gene clusters, we applied a more stringent cutoff of  $\log_2 = 1.5$  (an ~3-fold change in gene expression), but included all genes in operons in which at least one gene fits this description when comparing wild-type and *mftRA* or wild-

type cells grown in absence or presence of urate (Table 1); neighboring genes encoding potential (or confirmed) transcriptional regulators were also noted. In general, the fold-change in expression level was greater for early gene(s) in each operon. Increased expression of select genes in *mftRΔ* and in WT cells grown with 5 mM urate was validated by qRT-PCR (Fig. S5).

Several well-characterized gene clusters for which the corresponding metabolite(s) have been structurally and functionally identified were repressed by MftR and induced in presence of urate. A gene cluster annotated as two adjacent operons, *BTH\_I2357-2358* and *BTH\_I2359-2367*, was previously shown to encode proteins responsible for synthesis of burkholdacs, which are bicyclic depsipeptides that function as histone deacetylase inhibitors. These compounds were reported to accumulate on induction of a construct expressing the adjacent AraC family transcription factor encoded by *BTH\_I2369*, suggesting that this transcription factor functions as an activator.<sup>42</sup> This otherwise poorly expressed gene cluster was also reported to be induced in presence of trimethoprim.<sup>8</sup> Genes encoding proteins involved in synthesis of burkholdacs, including AraC, are markedly induced on deletion of ScmR, a LysR-family transcription factor recently reported to control expression of several biosynthetic gene clusters.<sup>11</sup> The *smcR* expression is increased 2-3-fold in *mftRΔ* or on addition of urate, whereas *mftR* expression is unaffected by deletion of ScmR, suggesting that MftR acts upstream of ScmR (Table S1).

Malleobactins, encoded by *BTH\_I2414-2426*, function as iron siderophores and the corresponding genes encoding NRPSs and iron siderophore transport proteins in *B. pseudomallei* were shown to be upregulated under iron-limiting conditions.<sup>43</sup> *BTH\_I2427* encodes a predicted extracytoplasmic function (ECF) sigma factor, which in *B. pseudomallei*

is involved in regulation of the malleobactin gene cluster. While the gene encoding the ECF sigma factor is upregulated ~5-fold in absence of MftR, no effect of urate was observed; expression of other genes in the malleobactin cluster was also less affected by urate addition than by absence of MftR, consistent with a role for ECF in gene regulation. We speculate that the inability of urate to induce expression may be linked to the affinity of MftR for the *ecf* gene promoter.

The antibacterial compound capistruin is a ribosomally synthesized 19-amino acid peptide that is post-translationally modified to generate a “lasso peptide” in which the C-terminal segment is threaded through a macrolactam ring formed by the nine N-terminal amino acids.<sup>44</sup> In *B. thailandensis*, the capistruin peptide precursor is encoded by *BTH\_I2437a* and the modifying enzymes by *BTH\_I2438-2440*. These genes are also repressed by ScmR.<sup>11</sup>

Most of the biosynthetic gene clusters under MftR control are encoded on chromosome II. A predicted pyoverdine biosynthetic cluster encoded by *BTH\_II0229-00234* was upregulated ~2-fold in *mftRA* and on addition of urate. Pyoverdines are the primary siderophores in *P. aeruginosa* and consist of a dihydroquinoline-type chromophore linked to a peptide. In *P. aeruginosa*, their production is under control of both an ECF sigma factor (the ortholog of *BTH\_I2427*) and quorum sensing.<sup>45</sup> The more significant upregulation on addition of urate compared to deletion of *mftR* suggests that urate causes upregulation by an additional mechanism, possibly by chelating iron and inducing an iron-limiting condition.

The siderophore pyochelin is composed of salicylate and cysteine-derived heterocycles, and it has lower affinity for iron compared to other compounds, including



pyoverdine.<sup>46</sup> In the iron-limited environment of a host, siderophores are important virulence factors, facilitating iron uptake by means of TonB-dependent receptors.<sup>47</sup> The *B. thailandensis* pyochelin gene cluster (*BTH\_II1823-1833*) is upregulated in *mftRΔ*, along with genes encoding TonB and the associated ExbB/ExbD transporter (*BTH\_II2024-2026*) and a TonB-dependent receptor encoded in an operon that also includes hemin transport proteins (*BTH\_II2139-2141*). This suggests that MftR controls expression of multiple iron-acquisition systems. To determine if *mftRΔ* cells indeed produce elevated levels of siderophores, we used a universal siderophore assay in which chromeazurol S and hexadecyltrimethylammonium bromide complexes with ferric iron to produce a blue color; in presence of a siderophore, the color will change from blue to orange. As shown in Fig. 4B, *mftRΔ* cells produced markedly higher levels of siderophores, whereas siderophore production returned to basal levels on complementation with plasmid-borne *mftR*.

Phenazines such as pyoverdine are redox active and may act as alternate electron acceptors, and their synthesis has been associated with reduced wrinkling during colony growth.<sup>48</sup> While wild-type *B. thailandensis* forms wrinkled colonies to maximize oxygen uptake through a larger surface area, *mftRΔ* cells formed smooth colonies, consistent with increased production of alternate electron acceptors (Fig. 5). Unexpectedly, *mftPΔfen1Δ* cells also exhibited a smooth colony morphology despite the increased biofilm formation indicated by clumping in liquid culture. This is reminiscent of the delayed wrinkling reported when *P. aeruginosa* is deleted for MexGHI-OpmD, the efflux system through which phenazines are exported;<sup>49</sup> a possible explanation for the smooth colony phenotype characteristic of *mftPΔfen1Δ* is therefore that MftP functions to efflux these compounds.

One of the both PKS- and NRPS-containing gene clusters encodes proteins involved in synthesis of bactobolins, some of which are potent antibiotics.<sup>50, 51</sup> Bactobolins are water-soluble compounds consisting of a C<sub>6</sub>-polyketide connected to a halogenated hydroxyl-valine. The bactobolin gene cluster (*BTH\_II1222-1242*) is conserved in *B. pseudomallei*, but absent from *B. mallei* and includes a transporter (encoded by *BTH\_II1241*) shown to be important for bactobolin export.<sup>50</sup> The crystal structure of bactobolin bound to a 70S ribosome suggests that bactobolin sterically interferes with P-site tRNA binding, thereby inhibiting peptidyl transfer.<sup>52</sup> Many genes in the bactobolin gene cluster are upregulated >20-fold in absence of MftR (Table 1 and Fig. S5).

Expression of genes encoding bactobolin biosynthetic proteins is regulated by BtaR2, a LuxR-type regulator encoded by *BTH\_II1231*.<sup>14</sup> The cognate AHL is synthesized by BtaI2 (*BTH\_II1227*), which is upregulated >15-fold in *mftRΔ* or on addition of urate. BtaI2 synthesizes two hydroxylated HSL, mainly *N*-3-hydroxy-decanoyl-HSL (3OHC<sub>10</sub>-HSL) and lower amounts of *N*-3-hydroxy-octanoyl-HSL (3OHC<sub>8</sub>-HSL). The *btaI2* gene is activated by BtaR2 in presence of either HSL. Global analysis of gene expression in a strain deleted for BtaR2 indicated that BtaR2 is dedicated to control of the bactobolin gene cluster.<sup>53</sup> Consistent with previous reports that bactobolins were particularly active against Gram-positive bacteria,<sup>54</sup> we observed a greater zone of inhibition in an agar well diffusion assay using cell-free culture supernatant of *mftRΔ* cells compared to WT (Fig. 4C).

The anti-proliferative polyunsaturated polyketide thailandamide is produced by proteins encoded by *BTH\_II1662-1676*, and the genes are under control of the orphan LuxR homolog BtaR5 encoded by *BTH\_II1681*.<sup>55</sup> Expression of genes encoding proteins involved in malleilactone biosynthesis (*BTH\_II2084-2099*) is also under control of an orphan LuxR

(BtaR4) that is encoded by *BTH\_II2087* and renamed MalR.<sup>56</sup> Malleilactone promotes virulence, as evidenced by prolonged survival of *Caenorhabditis elegans* fed *B. thailandensis* lacking an essential malleilactone biosynthesis gene.<sup>6</sup> The malleilactone gene cluster is normally silent, but is among the several gene clusters induced by trimethoprim.<sup>8, 56</sup>

The BtaI3-BtaR3 quorum sensing system (*BTH\_II0804-0805*), which is upregulated ~3-fold in *mftRΔ* (Table 1 and Fig. S5), is not encoded as part of a biosynthetic gene cluster. Inactivation of *btaR3* resulted in differential expression of 15 genes, most of which were not under control of MftR or urate.<sup>53</sup> We examined production of AHL using the *Agrobacterium tumefaciens* NTL4/pZLR4 reporter strain, which harbors a plasmid that confers AHL-sensitive production of β-galactosidase. Cell-free *B. thailandensis* supernatants were mixed with *A. tumefaciens* NTL4/pZLR4 in presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and the absorbance at 635 nm was recorded. As shown in Fig. 4D and S6, deletion of MftR resulted in increased AHL production whereas AHL production in cells complemented with pBBR-*mftR* was indistinguishable from that in WT cells.

Numerous HMAQs are produced by proteins encoded within the *hmaqABCDEFGH* operon (*BTH\_II1929-1935*).<sup>18</sup> All genes within this cluster were upregulated at least 6-fold in *mftRΔ* or on addition of urate (and verified by qRT-PCR; Fig. S5). In *B. ambifaria*, inactivation of genes within this cluster resulted in increased AHL production, while the opposite was reported on inactivation of a structural gene in *B. pseudomallei*, reflecting a complex QS network.<sup>18, 19</sup> SmcR was reported to activate expression of genes encoding HMAQs;<sup>11</sup> the increased expression of the *hmaq* gene cluster in *mftRΔ* may therefore be a consequence of increased *smcR* expression.

**Induction by trimethoprim.** As noted above, trimethoprim was identified as an inducer of certain biosynthetic gene clusters.<sup>8, 56</sup> A recent analysis of the *B. thailandensis* metabolome demonstrated that a large number of compounds accumulate when cells are exposed to trimethoprim,<sup>7</sup> suggesting that trimethoprim is a global inducer of secondary metabolite biosynthesis, much like urate is a global inducer of biosynthetic gene clusters that are repressed by MftR. In addition to bactobolins, capistrin, HMAQ, burkholdacs, malleilactone, and thailandamide, trimethoprim was shown to elicit production of terphenyl.<sup>7</sup> Terphenyl is produced by proteins encoded by *BTH\_II0204-0206*, a gene cluster that was modestly upregulated in absence of MftR. Additional cryptic gene clusters that were upregulated in absence of MftR or on addition of urate include *BTH\_II1952-1971* (encoding a predicted NRPS) and *BTH\_II1209-1219* (encoding both a predicted PKS and NRPS) immediately upstream of the bactobolin gene cluster; products associated with expression of these gene clusters have not been elucidated.

While the biosynthetic gene clusters that are upregulated in presence of urate correspond to the compounds that accumulate in cultures grown with trimethoprim, trimethoprim is unlikely to be direct inducer of the MftR regulon, as DNA binding by MftR is unaffected by the addition of trimethoprim (Fig. S7A). Trimethoprim is a folate biosynthesis inhibitor, and it inhibits enzymatic reactions that require tetrahydrofolate derivatives, including steps in purine biosynthesis. Under conditions of tetrahydrofolate deficiency, the biosynthetic intermediate 5-aminoimidazole-4-carboxamide-ribonucleotide (AICAR) was reported to accumulate and increased synthesis of both xanthine and adenine transporters was observed.<sup>57</sup> Since AICAR did not inhibit DNA binding by MftR either (Fig.

S7B), we speculate that other intermediates may accumulate or that an increased expression of purine transporters may lead to increased levels of inducing ligands for MftR.

**Model for gene regulation by MftR.** Successful host invasion is associated with activation of gene regulatory networks that operate together to control production of virulence factors and enhance fitness. Our data suggest that MftR functions at the top of a hierarchy of regulators that controls expression of genes linked to anaerobic respiration, T3SS, quorum sensing, and production of secondary metabolites. For example, MftR directly or indirectly activates expression of *bsaN*, and BsaN in turn activates expression of T3SS genes; MftR also activates *narL*, and NarL combined with NarX has been implicated in repression of the *arcDABC* gene cluster and activation of nitrate reductase genes (*nar*) (Fig. 6).

MftR also represses the gene encoding ScmR, which was recently reported to control biosynthetic gene clusters in response to quorum sensing signals.<sup>11</sup> Both MftR and ScmR repress *btaRI2*, however, the increased ScmR production expected in *mftRΔ* cannot overcome the derepression of *btaRI2* due to deletion of MftR. BtaRI2 in turn activates expression of the bactobolin biosynthetic gene cluster. Since AHL produced by BtaI2 functions to activate *scmR*, we cannot rule out that MftR-mediated repression of *scmR* is indirect and due to repression of BtaRI2 (Fig. 6). A similar pattern of gene regulation by MftR and ScmR pertains for *ecf*; both transcription factors repress *ecf*, yet the phenotype of the *mftR* deletion is *ecf* derepression, despite presumably increased ScmR levels in *mftRΔ*. ECF has been implicated in control of the malleobactin biosynthetic gene cluster. Likewise, increased repression of the *araC* gene linked to activation of the burkholdac biosynthetic gene cluster would be expected in *mftRΔ* if *araC* were regulated by ScmR only; however,

the opposite is observed, with increased *araC* expression in *mftRΔ*, indicating separate control of *araC* by the two transcription factors (Fig. 6). The malleilactone and thailandamide biosynthetic gene clusters were also reported to be under ScmR control, however, the genes encoding the cognate transcription factors, MalR and BtaR5, were not.<sup>11</sup> By contrast, both *malR* and *btaR5* are repressed by MftR, clearly indicating distinct regulatory pathways for MftR and ScmR in these cases.

The gene encoding ScmR is upregulated in response to increased cell density, and this results in activation of certain stress response genes and in repression of biosynthetic gene clusters. However, when an inducing ligand for MftR (urate) accumulates, derepression of biosynthetic gene clusters occurs; since exposure to purine metabolites such as urate would occur when bacterial infection elicits host xanthine oxidase activity, we suggest that differential expression of genes in the MftR regulon occurs to enhance bacterial fitness in the host environment. The concomitant induction of *scmR* may serve as an added layer of control to prevent overproduction of secondary metabolites under conditions of high cell density.

Sequencing of bacterial genomes have revealed that *Burkholderia* spp. harbor numerous biosynthetic gene clusters. These secondary metabolites have important roles as virulence factors, yet signals that trigger their production *in vivo* have proven difficult to identify. The discovery that the urate-inducible MftR functions as a global repressor of biosynthetic gene clusters and quorum sensing systems has implications for understanding activation of gene regulation networks during host invasion, and it paves the way for isolation of novel therapeutic scaffolds by upregulating cryptic natural product gene clusters. The pathology of infections with *Burkholderia* spp. includes the existence of

environments with limited oxygen supply, whether in the CF lung characterized by accumulation of mucus, in the abscesses that are associated with *B. pseudomallei* infections, or in the interior of biofilms. Evidently, MftR also controls expression of numerous genes whose products are required under anaerobiosis, suggesting a key role in adaptation to reduced oxygen environments and thereby virulence.

## Methods

Detailed procedures for construction of *mftRD*, verification of *mftRDfenID*, and construction of the complemented strains are described in the Supplemental Information, along with procedures for RNA isolation, RNA seq, data analysis, and qRT-PCR. Sequences of primers used are shown in Supplemental Tables S3 and S4. RNA-Seq data have been deposited to the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo>, under accession no. GSE100659.

Siderophore production was measured using chromazurol S (CAS) and acyl homoserine lactone production was measured using the indicator strain *Agrobacterium tumefaciens* NTL4/pZLR4, which harbors a plasmid that confers AHL-sensitive production of  $\beta$ -galactosidase. Survival under anaerobic conditions was assessed by incubating cultures in air-tight Hungate tubes. Effect of different compounds on DNA binding by MftR was assessed using electrophoretic mobility shift assays. Detailed procedures for all assays are available in Supplemental Information.

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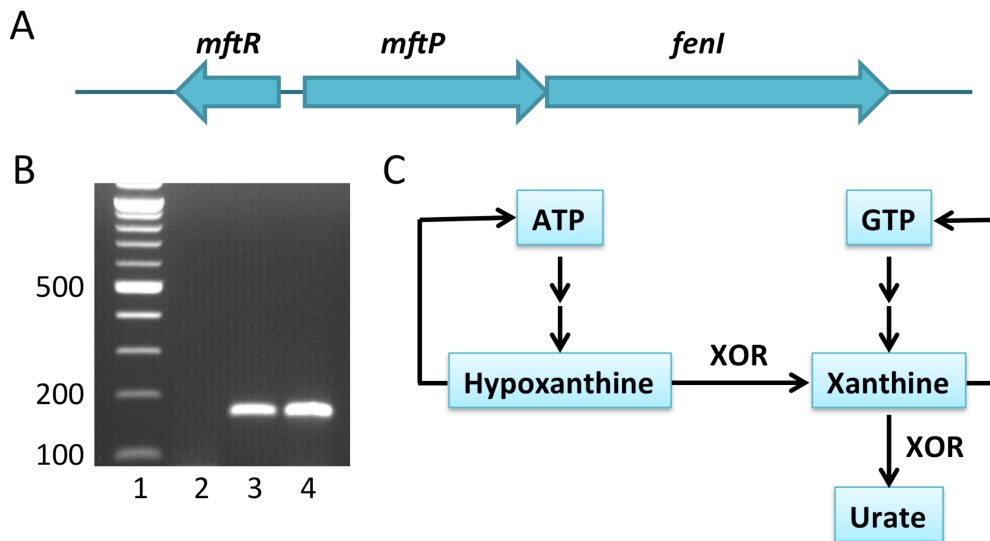


Figure 1. Urate is a ligand for MftR. A. The gene encoding MftR (*BTH\_I2391*; new locus tag *BTH\_RS24400*) is divergently oriented to an operon encoding the major facilitator superfamily transporter MftP and a predicted glycosyl hydrolase, FenI. B. Verification of *mftP-fenI* operon by PCR amplification of *mftP* fragment. Lane 1, Mw marker; lane 2, negative control using RNA as template; lane 3, positive control using genomic DNA as template; lane 4, cDNA synthesized using *fenI*-specific primer as template. C. Abridged version of the purine salvage pathway. Xanthine oxidoreductase (XOR) catalyzes conversion of hypoxanthine to xanthine to favor synthesis of GTP. Conversion of xanthine to urate diverts purines away from the salvage pathway. Hypoxanthine may be converted to ATP, while xanthine may be converted to GTP, both in several steps. The xanthine dehydrogenase (XDH) version of XOR primarily transfers substrate-derived electrons to  $\text{NAD}^+$  to generate NADH, whereas xanthine oxidase has decreased affinity for  $\text{NAD}^+$  and transfers electrons to molecular oxygen to produce reactive oxygen species.

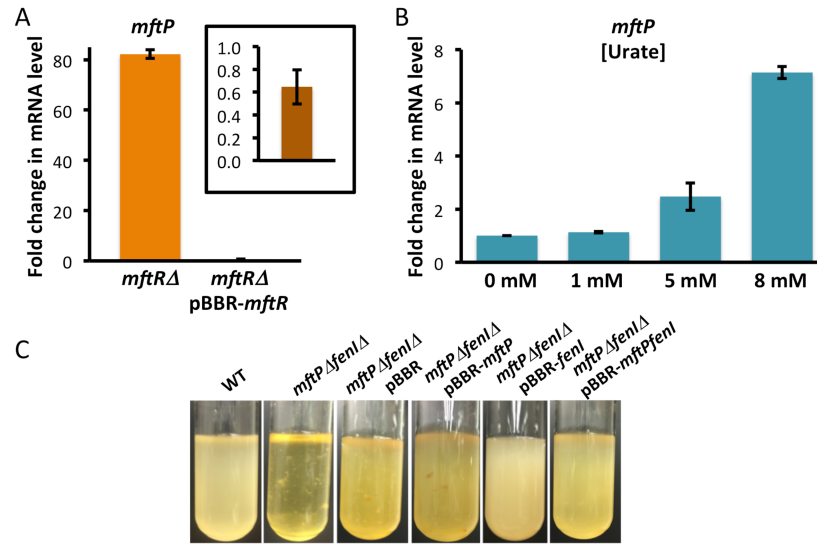


Figure 2. Complementation of *mftR*Δ and *mftP*Δ-*fenI*Δ strains. A. Change in *mftP* transcript levels relative to WT determined by qRT-PCR for *mftR*Δ and *mftR*Δ-pBBR-*mftR*. Inset shows relative transcript level in *mftR*Δ-pBBR-*mftR*. Transcript levels were calculated using  $2^{-\Delta\Delta C_T}$  relative to WT using reference gene GAPDH. Error bars represent standard deviation of three replicates. B. Gradual increase in *mftP* transcript levels with increasing concentrations of urate added to WT cells (1, 5, and 8 mM) calculated using  $2^{-\Delta\Delta C_T}$  relative to WT grown without urate. Error bars represent standard deviation of three replicates. C. Clumping in liquid culture resulting from deletion of *fenI*. Identified cultures were agitated just prior to capturing images, which are representative of three separate cultures.

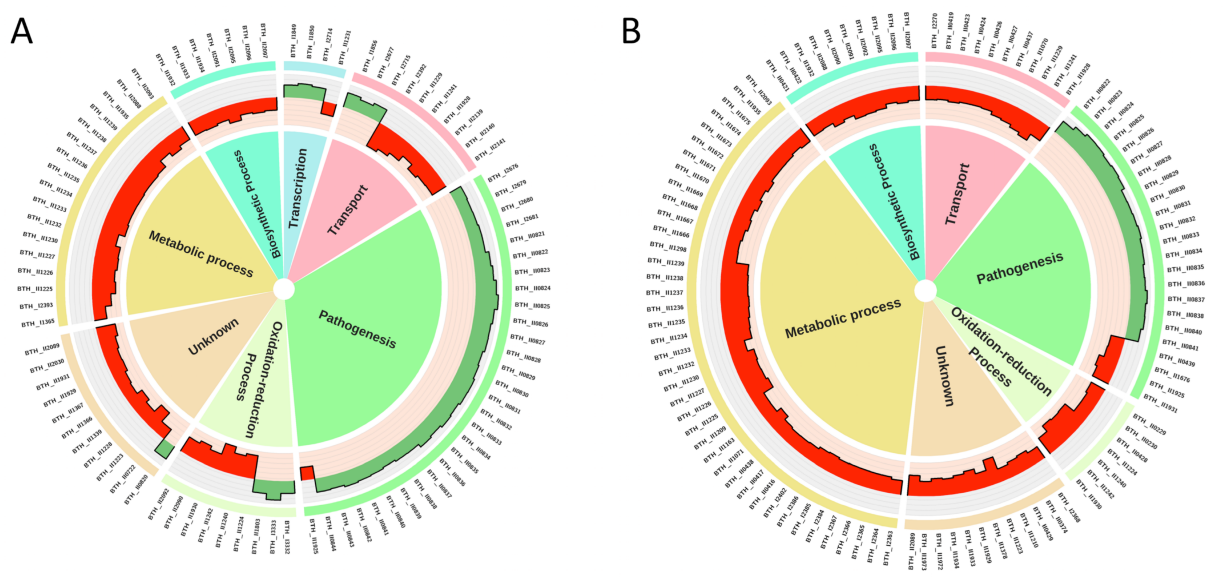


Figure 3. Circos plots showing differential gene expression. A. Higher levels of expression in wild-type compared to *mftRΔ* in green (genes activated by MftR) and reduced levels in red (genes repressed by MftR). Functional categories of genes identified in the central ring. B. Differential expression of genes in wild-type cells grown without or with 5 mM urate; genes repressed in presence of urate in green and genes induced by urate in red. Functional categories of genes identified in the central ring. The height of the bars corresponds to transcript level. Genes for which log<sub>2</sub>-fold change >|2| and P<0.05 are included.

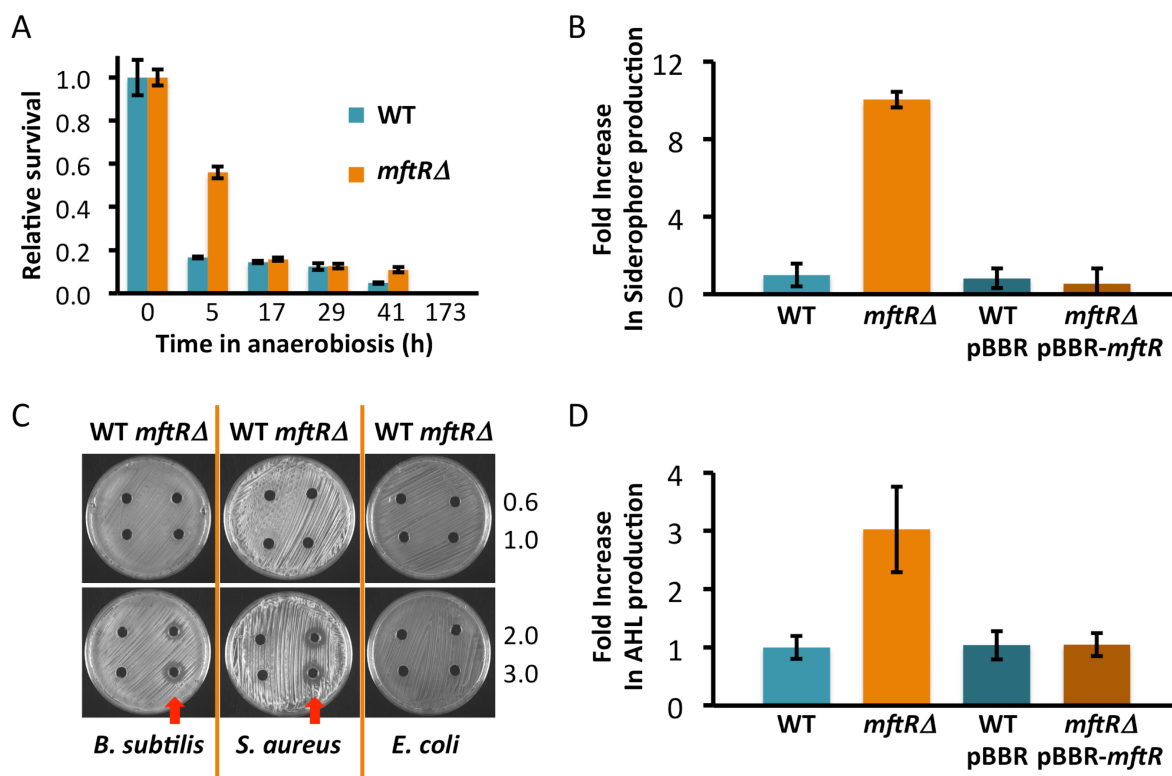


Figure 4. Phenotypes resulting from deletion of MftR. A. Survival under anaerobic conditions. Cells were transferred to airtight Hungate tubes at  $t = 0$  h; survival was determined by plating at the indicated times. Data are normalized to viability at  $t = 0$  h. Teal, WT; orange, *mftR*Δ. Error bars represent standard deviation of three replicates and are determined by standard propagation of errors. B. Siderophore production determined spectrophotometrically; data are represented as change in absorbance relative to control and normalized to absorbance change recorded for WT cells. Teal, WT; orange, *mftR*Δ; dark teal, WT-pBBR (empty vector); brown, *mftR*Δ-pBBR-*mftR*. Error bars represent standard deviation of three replicates. C. Detection of antibacterial compounds in cell-free supernatants. Supernatants were applied to holes in agar plates on which the indicated bacterial strains were spread. Culture supernatants were collected at OD<sub>600</sub> of 0.6, 1.0, 2.0,

and 3.0, as indicated at the right. For each set of plates, supernatant from WT was applied to the left and *mftRΔ* to the right. Clearing zones represent growth inhibition (arrows). D. AHL production determined spectrophotometrically; data are represented as change in absorbance relative to control and normalized to absorbance change recorded for WT cells. Color code as in (B). Error bars represent standard deviation of three replicates.

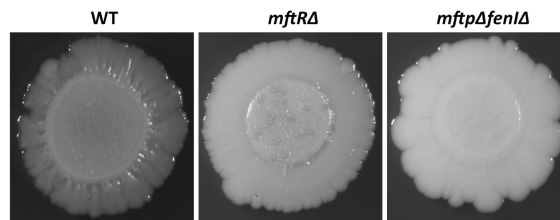


Figure 5. Colony morphology of mutant strains. Images were captured after incubation for ~5 days at 37 °C.

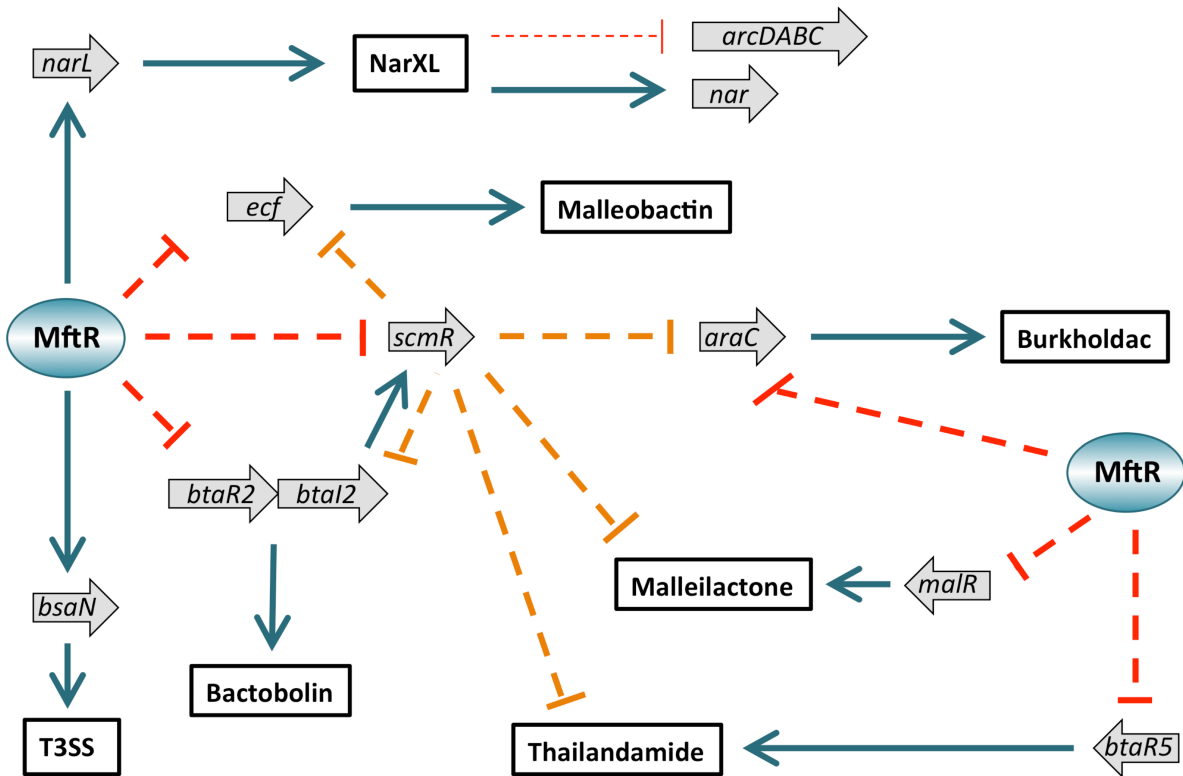


Figure 6. Model for gene regulation by MftR. Representative genes that are under MftR control are represented by grey block arrows. Activation is indicated by solid arrows (teal) and repression by dashed lines (red). End products are identified in bold frame. Thin line represents inferred regulation based on data from *P. aeruginosa*.

Table 1. MftR-controlled gene clusters encoding proteins with roles in quorum sensing and synthesis of secondary metabolites

Locus tag BTH_	Gene product / Secondary metabolite	log2 range <i>mftR</i> Δ/WT	log2 range WT(U)/WT(N)
I1952-1954 (+) I1955 (+) I1956-1967 (+) I1968-1971 (+)	NRPS (unknown product)	0.8 to 1.0 0.7 0.5 to 1.3 0.6 to 0.9	1.0 to 1.2 1.2 1.2 to 1.9 1.0 to 1.5
I2357-2358 (-) I2359-2367 (-) I2368 (-) I2369 (+)	<b>Burkholdac</b>  AraC	0.6 to 0.7 0.8 to 1.8 1.8 0.9	0.8 to 1.3 1.8 to 2.6 2.4 0.7
I2414 (-) 2415-2418 (-) I2419 (+) I2420 (+) I2421-2423 (-) I2424-2427 (-)	<b>Malleobactin</b>    ECF sigma factor	1.0 1.0 to 1.4 1.4 0.9 1.0 to 1.3 1.5 to 2.2	0.7 0.6 to 0.7 0.4 0.4 0.2 to 0.4 -0.2 to 0.1
I2437a (+) I2438-2440 (+)	<b>Capistruin</b>	1.5 0.9 to 1.2	1.3 0.4 to 0.9
II0229-0234 (+)	<b>Pyoverdine</b>	0.3 to 0.9	0.6 to 2.1
II0803 (-) II0804 (-) II0805 (-)	Oxidoreductase BtaI3 BtaR3	1.6 1.9 1.9	0.9 1.2 1.4
II1209-1219 (+) II1220-1221 (-) II1222 (+) II1223 (-) II1224 (-) II1225 (-) II1226-1228 (-) II1229-1230 (+) II1231 (-) I1232 (-) II1233-1241 (+) II1242 (-)	PKS/NRPS  <b>Bactobolin</b>    BtaI2  BtaR2	0.3 to 1.0 0.8 to 1.1 3.6 4.4 4.6 4.6 2.7 to 4.8 3.4 to 4.3 2.6 3.3 3.8 to 4.6 5.1	0.8 to 2.3 0.4 to 0.5 3.3 4.3 4.4 4.4 1.4 to 4.0 2.8 to 3.3 1.7 2.7 3.7 to 4.7 4.4
II1662-1672 (-) II1673-1674 (-) II1675-1676 (-) II1680-1681 (-)	<b>Thailandamide</b>   BtaR5	0.3 to 1.2 0.5 0.6 to 0.8 0.8 to 0.9	0.6 to 2.8 2.2 to 2.4 2.3 to 2.4 0.8

II1823 (-)	<b>Pyochelin</b>	1.6	0.5
II1824-1828 (-)		0.7 to 1.3	0.2 to 0.7
II1829 (-)		1.5	-0.3
II1830-1833 (+)		0.8 to 1.2	0.4 to 0.7
II1923-1924 (+)	Nitroreductase	1.7 to 2.1	0.1 to 0.3
II1925 (+)	Chitin-binding	2.2	2.9
II1926-1927 (+)	Reductase	0.9 to 1.3	0.8 to 1.6
II1928 (-)	Membrane protein	2.5	2.6
II1929-1935 (-)	<b>HMAQ</b>	2.6 to 2.9	2.5 to 3.3
II2084-2086 (-)	MalR (BtaR4)	0.3 to 1.2	0.4 to 0.8
II2087 (-)		1.8	1.2
II2088 (+)		3.1	2.3
II2089 (+)		3.7	3.4
II2090-2099 (+)	<b>Malleilactone</b>	1.6 to 3.3	1.6 to 3.0

Predicted operons according the *Burkholderia* Genome Database are indicated by a range of locus tags, with the coding strand identified by (+) and (-). Identified natural products encoded by the corresponding gene clusters are highlighted in boldface. Log2-fold changes show that MftR functions as a repressor in all instances and that urate induces expression. For annotation of each gene product, see Table S1.