Reporter-Guided Transposon Mutant Selection for Activation of Silent Gene Clusters in *Burkholderia thailandensis*

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Abstract

Most natural product biosynthetic gene clusters that can be observed bioinformatically are silent. This insight has prompted the development of several methodologies for inducing their expression. One of the more recent methods, termed reporter-guided mutant selection (RGMS), entails creation of a library of mutants that is then screened for the desired phenotype via reporter gene expression. Herein, we apply a similar approach to *Burkholderia thailandensis* and, using transposon mutagenesis, mutagenize three strains, each carrying a fluorescent reporter in the malleilactone (*mal*), capistruin (*cap*), or an unidentified ribosomal peptide (*tomm*) gene cluster. We show that even a small library of <500 mutants can be used to induce expression of each cluster. We also explore the mechanism of activation and find that inhibition of pyrimidine biosynthesis is linked to the induction of the *mal* cluster. Both a transposon insertion into *pyrF* as well as small molecule-mediated inhibition of PyrF triggers malleilactone biosynthesis. Our results pave the way toward the broad application of RGMS and related approaches to *Burkholderia* spp.

Graphical Abstract
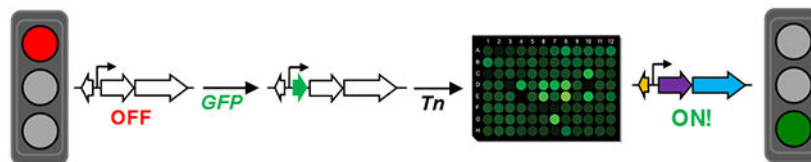
Silent biosynthetic gene clusters provide a treasure trove of new natural products. Herein, transposon mutagenesis is used in conjunction with fluorescent reporters to activate silent clusters, revealing nucleotide limitation as a key trigger for natural product biosynthesis in *Burkholderia thailandensis*.

Keywords

biosynthesis; *Burkholderia thailandensis*; natural products; silent gene cluster; transposon mutagenesis

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Microbial natural products remain a source of inspiration for chemists and biologists alike. The vast diversity of their structures and the specificity of interactions with their target sites are by now well-established.[1,2] Perhaps less-appreciated is the recent realization that the ~300,000 natural products reported thus far merely represent the tip of the iceberg, and that microbes harbor an untapped source of new secondary metabolites.[3–6] Indeed, microbial genome sequences that have accumulated over the past decade indicate a much larger potential for natural product biosynthesis than was previously imagined. Genomes of prolific producers routinely reveal >25 biosynthetic gene clusters (BGCs) for which a cognate secondary metabolite cannot be isolated.[4–6] While challenges associated with detection of secondary metabolites may explain this phenomenon in some cases, the current consensus is that the majority of BGCs are expressed at low levels in laboratory cultures. Referred to as "silent" or "cryptic", they outnumber those that are constitutively expressed by a factor of 5–10.[4,5] The presence of silent BGCs opens two major areas of inquiry: (1) how can they be activated and what kinds of compounds do they produce, and (2) how are they regulated and what conditions lead to their induction.

Several methods have been developed mostly to address the first question, including heterologous expression, insertion of constitutive or inducible promoters, co-culture, ribosome engineering, and high-throughput elicitor screening (HiTES).[7–14] Recently, the utility of modulating the expression of regulatory proteins either rationally or by leveraging random mutagenesis approaches has been demonstrated.[15–17] Guo et al. showed that silent BGCs can be activated in *Streptomyces* spp. using a reporter-guided UV mutagenesis approach referred to as RGMS (reporter-guided mutant selection), which led to the identification of a new glycosylated gaudimycin derivative.[17] Subsequently, transposon (Tn) mutagenesis was employed to connect genes, often unsuspected ones, to the production of a secondary metabolite in *Streptomyces albus and Streptomyces coelicolor*.[18,19] We recently expanded this strategy to *Burkholderia thailandensis* using phenotype-guided Tn mutagenesis to activate the expression of a silent type I polyketide synthase, thus enabling the discovery of the novel thailandenes.[20] Herein, we build on these results and implement reporter-guided Tn mutagenesis in *B. thailandensis* for inducing silent BGCs and identifying their underlying regulatory pathways. We demonstrate activation of three silent BGCs and in one case establish pyrimidine nucleotide limitation as a cue for induction of secondary metabolism.

B. thailandensis has emerged as a model system for exploring the production and regulation of silent secondary metabolite gene clusters.[5,21] It is a genetically tractable β-proteobacterium that carries ~25 BGCs, most of which are lowly-expressed. Currently, quorum sensing (QS) and ScmR are the key regulatory pathways implicated in controlling BGCs,[16,22,23] including those of malleilactone,[24–27] burkholdac,[28] and acybolin.[29] To globally explore pathways that are involved in activation of silent BGCs in *B. thailandensis*,

we set out to implement the approach shown (Fig. 1). A reporter gene (*lacZ* or *eGFP*) is inserted downstream of a silent promoter or within a silent cluster to provide an expression read-out. Subsequently, a Tn carrying a tetracycline-resistance marker (Tet[R]) is introduced to create a library of mutants. Those exhibiting resistance to Tet and enhanced LacZ activity or fluorescence are then examined further to determine the site of Tn insertion and production of the desired cryptic metabolite.

Our initial attempts to validate this approach used EZ-Tn5 mutagenesis in the sequence-defined in-frame *lacZ* insertion library in *B. thailandensis* pioneered by the Manoil group. [30] Because *lacZ* is encoded within a T8 transposon, subsequent mutagenesis led to frequent removal of the T8-derived reporter gene, rather than random insertion of Tn5. We therefore turned to genetically-encoded fluorescence proteins as reporters.[31] We first insured that their expression could be monitored rapidly by inserting *Burkholderia*-optimized *eGFP*, *eYFP*, or *RFP* into the bactobolin gene cluster (*bta*) as translational and transcriptional fusions (see SI, Tables S1, S2). The *bta* cluster is QS-activated and therefore expressed at high cell densities.[32–35] Growth of *bta-eGFPx2*, *bta-eYFPx2* and *bta-RFPx2* followed by imaging by fluorescence microscopy showed this to be the case (Fig. 2A–C). The double reporter-constructs increased signal-to-noise and allowed us to easily monitor fluorescence emission of bacterial cultures in grown in LB using a plate reader, thus simplifying the expression read-out. Next a double reporter cassette was inserted into three silent BGCs coding for malleilactone (*mal*),[24–27] capistruin (*cap*),[36,37] and a yet-uncharacterized putative TOMM (thiazole-oxazole modified microcin, *tomm*) (Fig. 3A).[38] With *mal* and *cap*, we generated translational fusions, while with *tomm* a transcriptional fusion was introduced directly downstream of an identifiable promoter that precedes *tommR*. We used different fluorophores for each locus (*mal-eGFPx2*, *cap-YFPx2*, P_{tomm}-*RFPx2*), with the idea of conducting this screen simultaneously with multiple unknown silent clusters in the future, if the method was successful.

Each of the three reporter strains was then subjected to Tn mutagenesis, in which a modified Tn8, carrying a tetracycline resistance marker (Tet[R]), was inserted using conjugation with the appropriate *E. coli* donor strain.[39] The transformants were selected on Tet-agar plates and a total of 200–500 colonies arrayed into 96-well plates. Subsequently, the plates were grown for a defined period and absorbance at 600 nm (OD_{600}) and fluorescence emission were measured as a proxy for growth and expression of the chosen cluster, respectively. Results for all three clusters are shown (Fig. 3B–3D). All three clusters were expressed at low levels in the absence of any mutation. Within the small library generated, growth defects were rare as less than 10% of the mutants exhibited an OD_{600} value >2-fold lower (or higher) than the unmutagenized reporter strain. Even with a relatively small number of transformants, we could observe induction of each gene cluster, which we arbitrarily defined as a >5-fold (*mal, cap*) or >7-fold (*tomm*) expression in the transfor-mant, as determined by fluorescence emission spectroscopy. With the *mal* cluster, we found 2 hits from 160 transformants. Likewise, with *cap* and *tomm* we found 5 hits (from 480) and 10 hits (from 320), respectively, suggesting that activation of silent pathways via Tn mutagenesis was fairly efficient.

To verify that each cluster was in fact induced, selected hit(s) were assessed by RT-qPCR and/or HPLC-MS. The product of the *mal* cluster is known, but insertion of the *eGFP* reporter genes rendered the strain deficient in malleilactone production, as experiments with trimethoprim, a proven inducer of the *mal* cluster,[40] demonstrated. We therefore transplanted the Tn from the selected strain into a wt background using established methods. [41] Comparison of the secondary metabolomes of wt and the Tn-carrying strain demonstrated 26-fold enhanced malleilactone production (Fig. 4A). For the *cap* cluster, three mutants were examined by RT-qPCR, revealing moderate upregulation in *capB* relative to wt for two strains (Fig. 4B). All three Tn mutants did produce capistruin, as determined by HPLC-Qtof-MS (Fig. 4C). In contrast, no capistruin was detected in the wt strain. Lastly, mutants that exhibited increased expression of the *tomm* cluster were further verified by RT-qPCR. Four selected mutants showed a 2–4.5-fold upregulation of *tommR* relative to wt (Figs. 3A, 4D). The product of the *tomm* cluster is not yet known. Repeated attempts to identify it proved unsuccessful. Nonetheless, our results verify that all three silent clusters were induced using the workflow described in Fig. 1, and that a combination of reporter screening and Tn mutagenesis can activate silent BGCs in a targeted fashion in *B. thailandensis.*

We next investigated the site of Tn insertion in selected hits to better understand the origin of induction of the *mal*, *cap*, and *tomm* clusters. Using arbitrary PCR, we identified *pyrF* as the site of Tn insertion in the mutant that induced the *mal* cluster most (Fig. 3B). PyrF catalyzes the final step in UMP biosynthesis,[42] a pathway that intersects with one-carbon metabolism, the inhibition of which by trimethoprim has been shown to induce malleilactone synthesis.[40] Examination of three mutants that increased *cap* expression identified a putative Ser/Thr kinase (BTH_I0113), a *barD* homolog (BTH_II0562), and a gene of unknown function (BTH_I2853) as genes that control or affect the production of capistruin (Fig. 3C). Lastly, examination of select mutants identified in the *tomm* screen revealed Tn insertion into ribonuclease T2 (BTH_II1789), a flagellar hook protein (FlgE), glucose-1-phosphate thymidylyltransferase (BTH_I1470), and a gene of unknown function (BTH_I0403), all previously not known to control secondary metabolism. How these genes contribute to the regulation of secondary metabolism remains to be determined. The results show that control over natural product biosynthesis is complex and that many unsuspected genes are involved in this process. Xu et al. recently reached a similar conclusion by examining genes that contribute to actinorhodin biosynthesis in *Streptomyces coelicolor*. [18]

The results above suggest a connection between pyrimidine nucleotide biosynthesis and malleilactone production. As independent corroboration, we conducted experiments with 5-fluoroorotic acid (5-FOA), a well-known competitive inhibitor of *E. coli* PyrF,[42] which is homologous to the enzyme in *B. thailandensis*. Treatment of wt *B. thailandensis* with 5-FOA revealed dose-dependent overproduction of malleilactone (Fig. 5A). Optimal production was observed at 100 µM 5-FOA, leading to 12-fold enhanced malleilactone biosynthesis. Moreover, much like trimethoprim, 5-FOA globally affected secondary metabolism as amplified production of numerous secondary metabolites – including several hydroxymethyl-quinoline (HMQ) derivatives[29,43] and thailandamide[44] – was also observed (Fig. 5A). These results provide a link between pyrimidine biosynthesis and malleilactone biogenesis that would have been difficult to predict a priori.

Trimethoprim induces the *mal* cluster by activating *malR*, an 'orphan' LuxR regulator that serves as a positive transcriptional regulator.[40,45] We examined the involvement of *malR* in the activation of *mal* using RT-qPCR and found its expression to be ~2-fold enhanced in *pyrF::Tn* relative to wt (Fig. 5B). Moreover, addition of 5-FOA to wt cells also resulted in ~2-fold up-regulation of *malR* and *malF* (Fig. 5B). At the same time, a markerless *malR* deletion mutant (*malR*) failed to trigger malleilactone synthesis in response to 5-FOA (Fig. S1). Thus, inhibition of pyrimidine biosynthesis by 5-FOA leads to enhanced *malR* expression, which in turn induces the *mal* cluster. These results consolidate the link between pyrimidine biosynthesis and cryptic metabolism in *B. thailandensis*.

In summary, we report the implementation of RGMS for induction of silent BGCs in *B. thailandensis*. This approach was previously used in *Streptomyces* sp. R435, where UV mutagenesis along with a promoter-reporter construct led to activation of a gaudimycin gene cluster.[17] Meanwhile Xu et al. and Ahmed et al. used Tn mutagenesis with either a compound-specific colorimetric read-out (actinorhodin, *S. coelicolor*) or a reporter gene in *S. albus*, respectively, to investigate genes involved in the regulation of secondary metabolite biosynthesis.[18,19] We have similarly used Tn mutagenesis along with cluster-specific reporter constructs to induce silent BGCs. By applying the method to three different BGCs, we demonstrate the cluster-agnostic feature of this approach. Following up on one Tn mutant, we show that alteration of pyrimidine biosynthesis, either via gene deletion or by small molecule inhibition, results in upregulation of the *mal* BGC. Our previous work showed that Tmp and sulfonamide antibiotics, inhibitors of one-carbon metabolism, also result in malleilactone biosynthesis.[45] Thus, using two very different approaches, we have converged on the same primary metabolite network, the inhibition of which is tied to induction of secondary metabolism. In the current study, we used a small number of Tn mutants to activate the selected operons. Exhaustive Tn mutagenesis could identify all genes that are involved in regulating, or indirectly affecting the expression of, these and other silent BGCs. RGMS is an addition to the cadre of approaches available for gaining further insights into the hidden metabolome of *B. thailandensis*. Much like HiTES, reporter-guided Tn mutagenesis can address both the chemistry, i.e. small molecule products, and biology, i.e. regulatory networks, that underlie silent BGCs. And much like HiTES, read-outs other than transcriptional/translational reporters may be employed, for example bioactivity assays or mass spectrometry.[46–49] Coupling these additional read-outs to Tn mutagenesis is currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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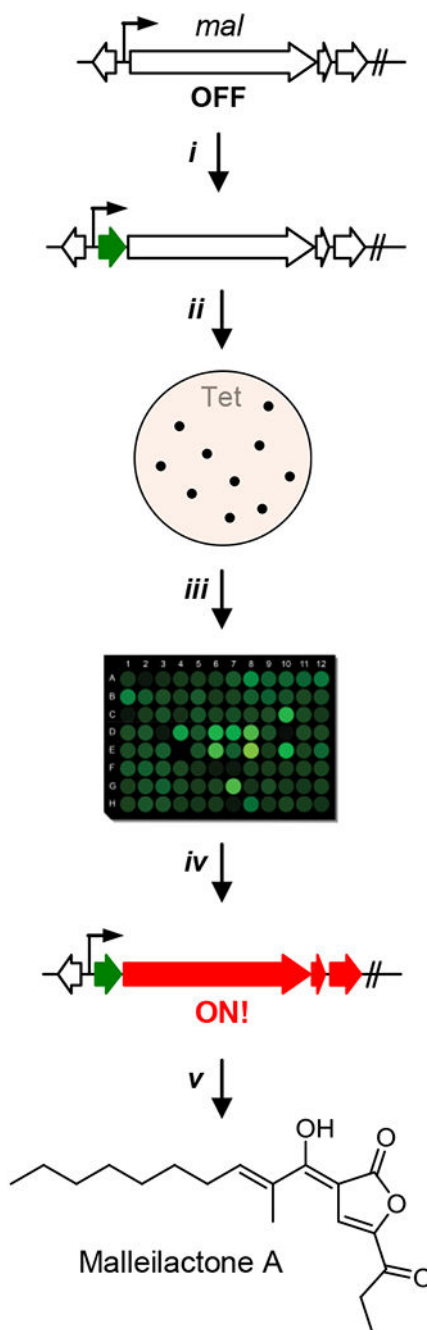
Figure 1.
Workflow for reporter-guided Tn mutant selection. A reporter gene such as GFP (shown in green) is introduced into a silent BGC (*i*). The resulting strain is subjected to Tn mutagenesis and Tn-carrying strains isolated on selective media (*ii*). Fluorescence emission is measured as a proxy for expression of the silent cluster (*iii*). Appropriate mutant(s) that show enhanced expression of the BGC are used to characterize the cryptic metabolite (*iv, v*).
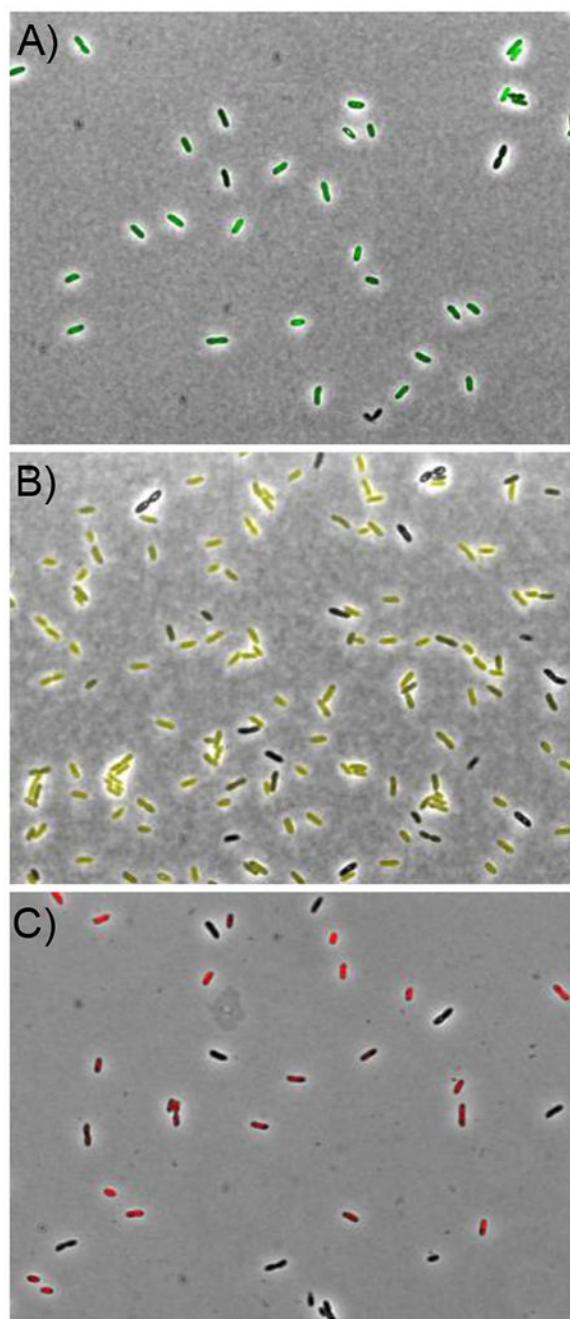
Figure 2.
Secondary metabolite gene expression detected with fluorescence reporters. Expression of the QS-activated *bta* cluster with a double reporter cassette containing *eGFP* (A), *eYFP* (B), or *RFP* (C).
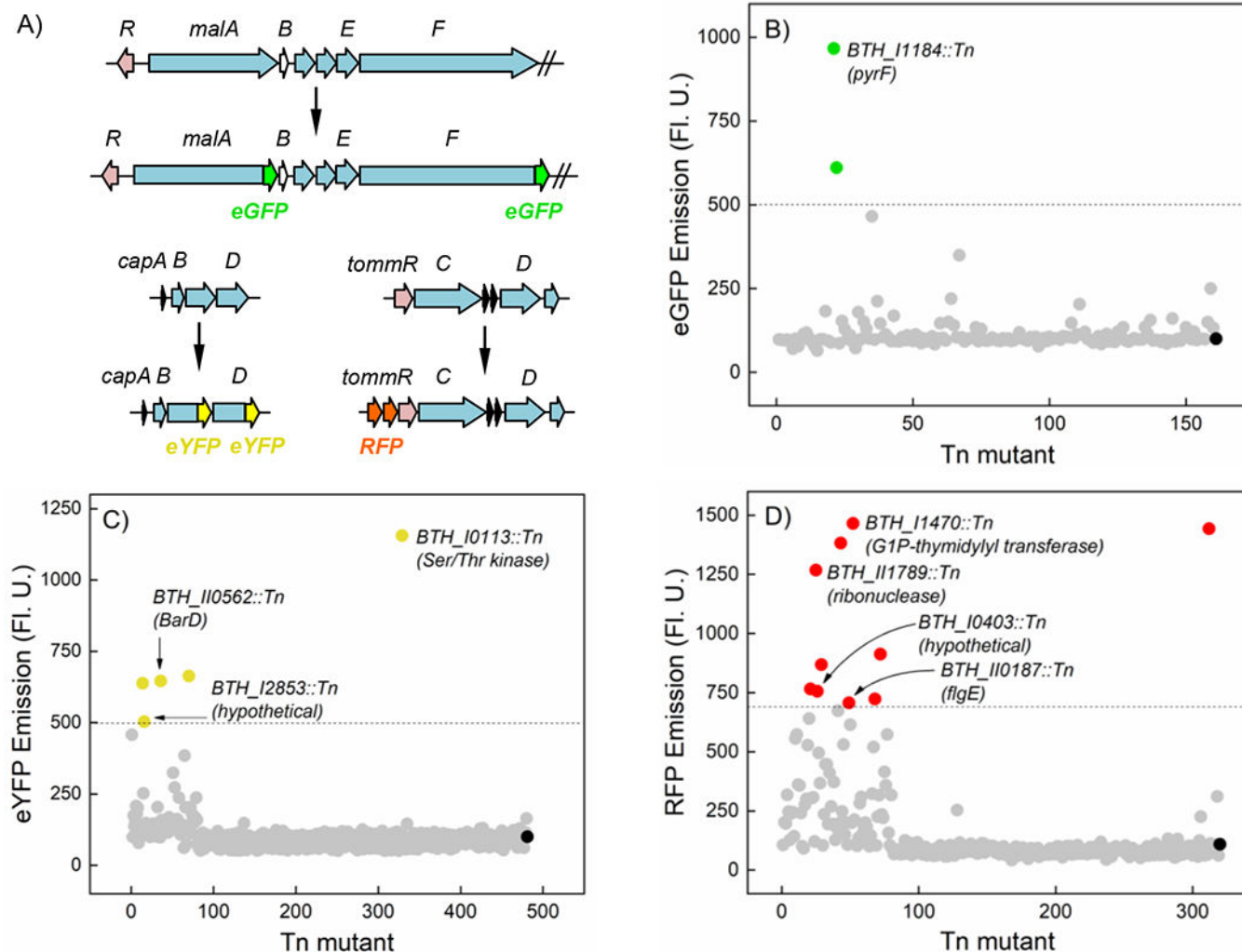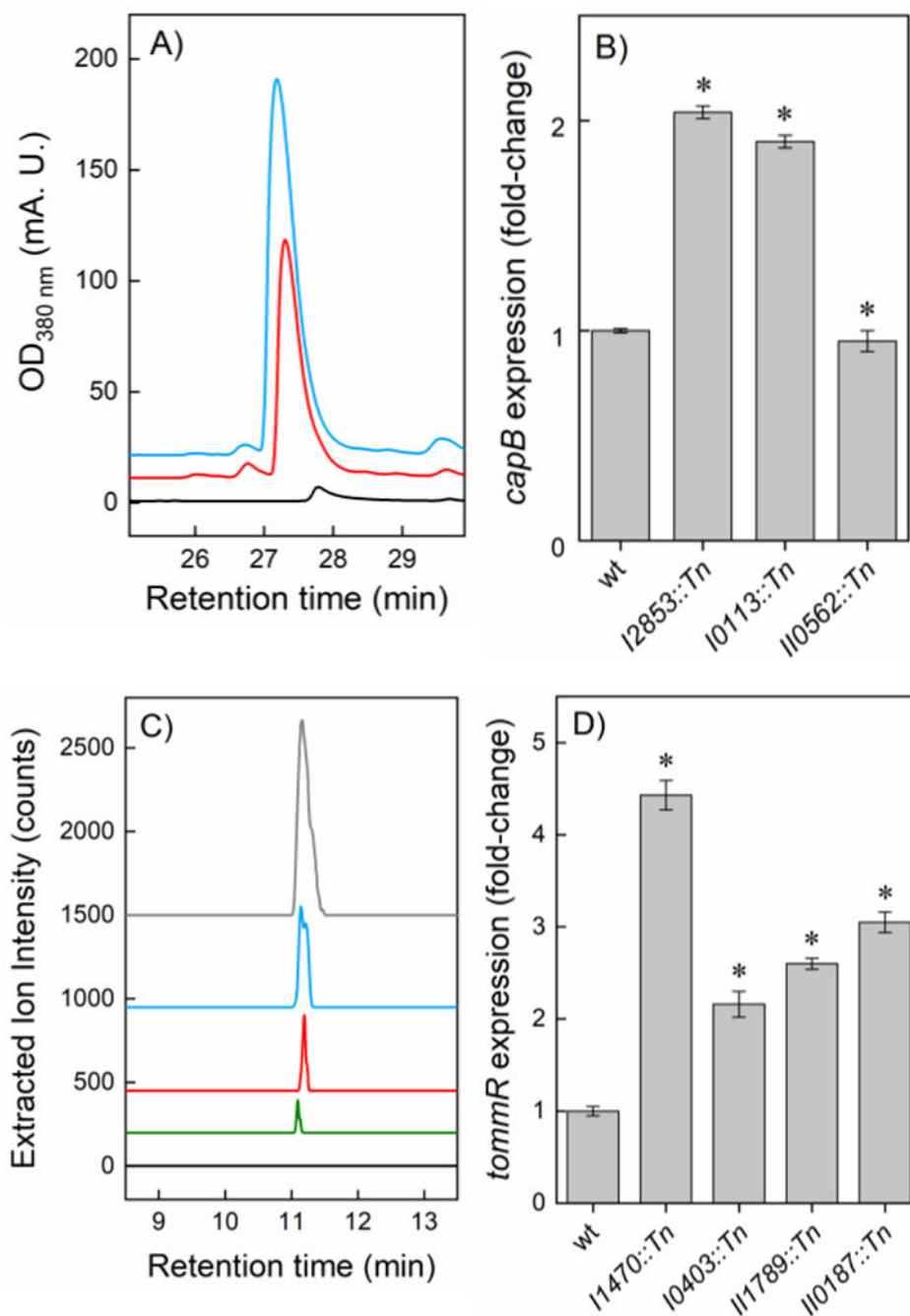
Figure 3.
Reporter-guided Tn mutant selection in *B. thailandensis*. (A) Reporter constructs generated to induce the silent *mal*, *cap*, and *tomm* BGCs. Gene are color-coded as follows: blue, biosynthetic enzyme; purple, regulatory protein; black, precursor peptide, white, gene of unknown function. (B-D) Activation of the *mal* (B), *cap* (C), and *tomm* (D) BGCs. The fluorescence output is normalized to the unmutagenized reporter which is shown as a block sphere in each case. Mutants above the dotted lines were designated as hits. Mutants in which the site of Tn insertion was successfully identified using arbitrary PCR are labelled.

Figure 4.
Validation of Tn mutants. (A) HPLC-MS analysis of wt (black trace) and *pyrF::Tn* (red) reveals enhanced malleilactone production, similar to levels obtained with trimethoprim-treated wt cells (blue). (B) RT-qPCR analysis shows enhanced *capB* expression in the indicated Tn mutants. (C) HPLC-MS analysis of wt (black trace), *BTH_I0113::Tn* (green), *BTH_II0562::Tn* (red), and *BTH_I2853::Tn* (blue) reveals capistruin production only in the Tn mutants. A capistruin standard (gray) is also shown. (D) RT-qPCR analysis shows increased *tommR* expression in the indicated Tn mutants. Data in panels B and D represent

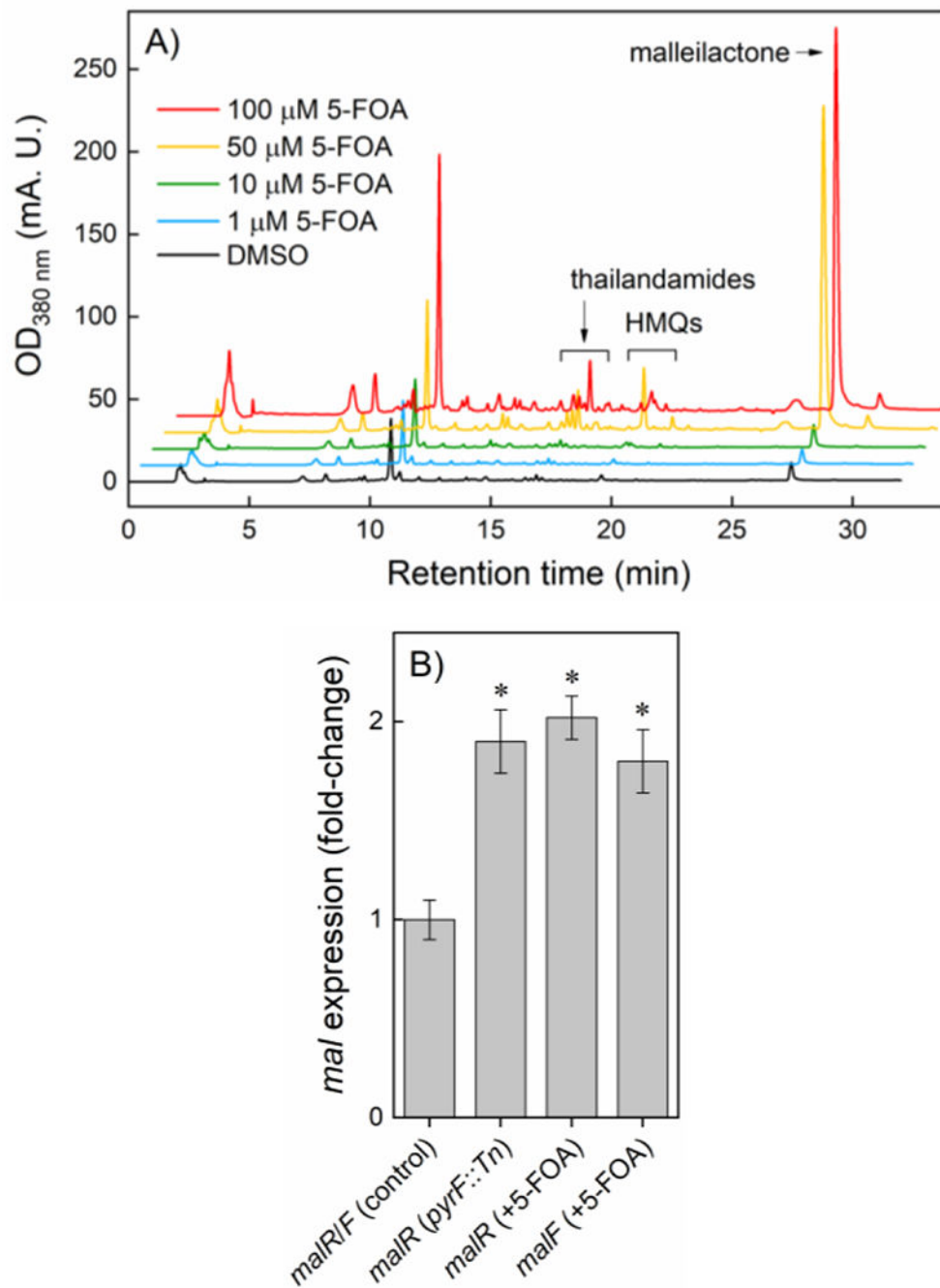the averages of two independent measurements ± standard error; p-values (*, <0.01) are indicated.

Figure 5.
5-Fluoroorotic acid broadly activates secondary metabolism in *B. thailandensis*. (A) HPLC-MS analysis of *B. thailandensis* treated with DMSO (control) or with 5-FOA at the concentrations indicated. Identified metabolite groups are labeled. (B) RT-qPCR analysis shows induction of *malR* after inhibition of PyrF either by gene deletion or by 5-FOA. Data represent the averages of three independent measurements ± standard error; p-values (*, <0.01) are indicated.