A Unique Tryptophan C-Prenyltransferase from the Kawaguchipeptin Biosynthetic Pathway

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Abstract: Cyanobactins are a rapidly growing family of linear and cyclic peptides produced by cyanobacteria. Kawaguchipeptins A and B, two macrocyclic undecapeptides reported earlier from Microcystis aeruginosa NIES-88, are shown to be products of the cyanobactin biosynthetic pathway. The 9 kb kawaguchipeptin (kgp) gene cluster was identified in a 5.26 Mb draft genome of Microcystis aeruginosa NIES-88. We verified that this gene cluster is responsible for the production of the kawaguchipeptins through heterologous expression of the kgp gene cluster in Escherichia coli. The KgpF prenyltransferase was overexpressed and was shown to prenylate C-3 of Trp residues in both linear and cyclic peptides in vitro. Our findings serve to further enhance the structural diversity of cyanobactins to include tryptophan-prenylated cyclic peptides.

Cyanobactins are a family of ribosomally-synthesized and posttranslationally modified peptides (RiPPs) produced by cyanobacteria.[1–6] Cyanobactin posttranslational modifications include N-to-C macrocyclization; epimerization; heterocyclization to form thiazolines and oxazolines; oxidation of heterocycles to thiazoles and oxazoles; N-methylation of His; O-prenylation on Ser, Thr, and Tyr; and N-prenylation.[2,7,8] Biological activities that have been reported for cyanobactins include anticancer, antimalarial, antibacterial, and protease inhibitory activity.[2]

The posttranslational prenylation of cyanobactins is catalyzed by a prenyltransferase enzyme encoded within the cyanobactin biosynthetic gene cluster, which uses 3-methylbut-2-en-1-yl group derived from dimethylallyl pyrophosphate (DMAPP).[9–12] Although a putative prenyltransferase gene is present in all known cyanobactin gene clusters, only a few of the cyanobactins are known to be prenylated, including prenylagaramides, aetuxamides, trunkamides, and anacyclamides.[4,6,10,12] The known cyanobactin prenyltransferases are O-prenyltransferases that catalyze the O-prenylation of Tyr, Thr, and Ser in forward or reverse orientation. C-prenylated cyanobactins have been shown to be synthesized originally as O-prenylated peptides that later undergo a Claisen rearrangement to yield C-prenylated peptides.[12]

Kawaguchipeptins are macrocyclic undecapeptides produced by the cyanobacterial strain Microcystis aeruginosa NIES-88.[10,14] Two variants of kawaguchipeptin have been reported (Scheme 1). Kawaguchipeptin A contains two C-3-prenylated tryptophan residues and a δ-Leu residue.[13] Kawaguchipeptin B consists of solely unmodified amino acids and is reported to show antimicrobial activity against Staphylococcus aureus.[14] Herein, we report a genome sequence for Microcystis aeruginosa NIES-88, identify the kawaguchipeptin biosynthetic gene cluster, and confirm enzymatic prenylation activity.

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These are not the final page numbers!
The kawaguchipeptin biosynthetic pathway in *Microcystis aeruginosa* NIES-88.

We obtained a 5.26 Mb genome sequence for *Microcystis aeruginosa* NIES-88 by using a combination of 3 kb 454 sequencing and short-insert illumina Miseq paired-end data, which were subsequently assembled into 29 scaffolds. A total of 4,996 genes, including 4 ribosomal RNA operons and 41 tRNAs, were annotated from this genome. The kawaguchipeptin precursor gene was identified through tBLASTn using the predicted kawaguchipeptin peptide backbone. The genome sequence contained 4,996 genes, including 4 ribosomal RNA operons and 41 tRNAs, which were subsequently assembled into 29 scaffolds. A total of 4,996 genes, including 4 ribosomal RNA operons and 41 tRNAs, were annotated from this genome. The kawaguchipeptin precursor gene was identified through tBLASTn using the predicted kawaguchipeptin peptide backbone (WLNGDNNWSTP). The KgpE precursor peptide was found to encode three exact copies of the WLNGDNNWSTP core (Figure 1). The KgpE precursor peptide was encoded in a single operon, that show homology to genes present in other known cyanobactin gene clusters. The grey arrows indicate genes that encode proteins with unknown functions.

To demonstrate that the *kgp* operon in *E. coli* demonstrates that the *kgpA-G* genes confer the production of 1 and 2 in vivo. Extracted ion chromatographs of LC–HRMS analysis of: Authentic 1 and 2 from *M. aeruginosa* NIES-88 (1, 2); 2 in *E. coli* transformed with pDK-kgp1 (3); 1 in *E. coli* transformed with pDK-kgp1 (4); 1 in *E. coli* co-transformed with pDK-kgp1 and pMBI and supplied with mevalonolactone 3 (5); and 2 in *E. coli* co-transformed with pDK-kgp1 and pMBI and supplied with mevalonolactone 3 (6). The experiments establish that the *kgpA-G* genes confer TOP10. Liquid chromatography with high-resolution mass spectrometry (LC–HRMS) guided metabolite profiling of *E. coli* cells transformed with pDK-kgp1 revealed the presence of 2, for which the LC retention time and HRMS profile matched authentic 2 isolated from *M. aeruginosa* NIES-88 (Figure 2, traces 2–3, and Figure S2). *E. coli* cells carrying pDK-kgp1 alone, however, did not produce 1 (Figure 2, trace 5), which is potentially derived from 2 by bisprenylation at C-3 of Trp by prenyltransferase KgpF. We hypothesized that this observation may have been due to the lack of sufficient endogenous prenyl donor dimethylallyl pyrophosphate (DMAPP) in *E. coli*. To overcome this problem, we co-transformed *E. coli* with pDK-kgp1 and the plasmid pMBI, which harbors four yeast mevalonate-dependent isoprenoid pathway biosynthetic genes that can convert mevalonate to isopentenyl pyrophosphate (IPP), a precursor to DMAPP.

Co-expression of the pDK-kgp1 and pMBI genes in *E. coli* TOP10 supplied with exogenous mevalonolactone 3 (1.0 mM) led to the production of both 1 and 2 that matched their authentic standards from *M. aeruginosa* NIES-88 (Figure 2, traces 1–2, 6–7, and Figure S3), as assessed by LC–HRMS.

These experiments establish that the *kgpA-G* genes confer...
1 and 2 biogenesis in vivo. In addition, the coexistence of 1 and 2 in *E. coli* cells transformed with both pDK-kgp1 and pMBI and the lack of 1 in *E. coli* cells transformed with pDK-kgp1 alone strongly suggest that 2 is the direct biosynthetic precursor to 1 and bis-prenylation by KgpF is likely the last enzymatic step in the biosynthetic maturation of kawaguchipeptins.

We overexpressed and purified the recombinant KgpF from *E. coli* and assessed the enzymes ability to process a range of cyclic and linear peptides and to use isopentenyl pyrophosphate (IPP), DMAPP, and geranyl pyrophosphate (GPP; Table 1 and Figures S4–S24).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cofactor</th>
<th>Mono-prenylation</th>
<th>Di-prenylation</th>
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<tbody>
<tr>
<td>Cyclic [WLNGDNWSTP] (2)</td>
<td>DMAPP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cyclic [WLNGDNWSTP] (2)</td>
<td>IPP</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cyclic [WLNGDNWSTP] (2)</td>
<td>GPP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cyclic [TSQWGSPV] (4)</td>
<td>DMAPP</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>Cyclic [SAQWQNGFVP] (5)</td>
<td>DMAPP</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>Cyclic [HAFICGDQDPTCYP] (6)</td>
<td>DMAPP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cyclic [LGIMHMP] (8)</td>
<td>DMAPP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WLNLDNWWSTP (9)</td>
<td>DMAPP</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>WLNLDNNWSTPAYDG (10)</td>
<td>DMAPP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EDWYFDPAYDG (11)</td>
<td>DMAPP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VPWPPAYDG (12)</td>
<td>DMAPP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Boc-Trp (13)</td>
<td>DMAPP</td>
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<td>Boc-Trp (13)</td>
<td>IPP</td>
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<td>Boc-Trp (13)</td>
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<tr>
<td>Boc-Trp (14)</td>
<td>IPP</td>
<td>-</td>
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</tbody>
</table>

[+] Product detected, [-] Product not detected.

Our results show that the enzyme processes a second Trp residue within macrocyclic peptide substrate 2, whereas in the linear peptide 9, despite 40 h of incubation, only a single modification was observed with DMAPP as the cofactor (Table 1; Figures S5–S7,S18–S20). The catalytic activity of the enzyme decreased when IPP was used as a cofactor instead of DMAPP. Interestingly, the enzyme could not use GPP as a cofactor. According to these results, the selectivity of prenylation in linear substrates can be explained by the necessity of the Trp residue to be sandwiched between two residues. Terminal Trp residues would presumably be too strongly solvated to bind efficiently the enzymatic site and undergo transformation (Figures S20, S22).

O- or C-prenylation of Tyr, Ser, and Thr in forward or reverse orientation have been observed for cyclic cyanobactins.[2,5,12] C-prenylated peptides like the C-prenylated Tyr in astuararamides have been reported.[15] However, the latter is the result of reverse O-prenylation on the oxygen atom of Tyr followed by Claisen rearrangement.[13] The biochemical characterization of C-3 Trp prenylation[11] and demonstration of the presence of a homologue of the prenyltransferase gene *kgpF* in the gene cluster confirm that this is a direct posttranslational modification, which is rare in cyanobactins. To our knowledge, C-3 prenylation of Trp through posttranslational modification of a peptide has been demonstrated only once before for the ComX peptide, a pheromone produced by *Bacillus subtilis* and related bacilli.[17,18]

Trp prenylation is common in some plants and bacteria, but mostly in fungi, and the respective prenyltransferases catalyze the addition of a dimethylallyl group to Trp during the synthesis of secondary metabolites.[19,20,21] The synthesis and biosynthesis of these compounds, particularly indole alkaloids that contain prenylated Trp at their core, have been the subject of considerable interest.[22] Our findings therefore expand the chemical diversity of cyanobactins and confirm the existence of a rare Trp prenyltransferase. The prenyltransferases of the cyanobactin family are now known to catalyze the O-, C-, and N-prenylation of amino acids in cyclic peptides.

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[23] The draft genome of *Microcystis aeruginosa* NIES-88 has been deposited in GenBank under the accession number JXYX00000000.

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