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A Unique Tryptophan C-Prenyltransferase from the Kawaguchipeptin Biosynthetic Pathway

Anirudra Parajuli[+], Daniel H. Kwak[+], Luca Dalponte[+], Niina Leikoski, Tomas Galica, Ugochukwu Umeobika, Laurent Trembleau, Andrew Bent, Kaarina Sivonen, Matti Wahlsten, Hao Wang, Ermanno Rizzi, Gianluca De Bellis, James Naismith, Marcel Jaspars, Xinyu Liu, Wael Houssen,* and David Peter Fewer**

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, aestuaramides, 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.[13,14] Two variants of kawaguchipeptin have been reported (Scheme 1). Kawaguchipeptin A contains two C-3-prenylated tryptophan residues and a D-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.

[*] A. Parajuli,[+] Dr. N. Leikoski, T. Galica, Prof. K. Sivonen, M. Wahlsten, Dr. H. Wang, Dr. D. P. Fewer
Microbiology and Biotechnology Division
Department of Food and Environmental Sciences
P.O.Box 56, Viikki Biocenter, Viikinkaari 9, 00014
University of Helsinki (Finland)
E-mail: david.fewer@helsinki.fi

D. H. Kwak,[+] Prof. X. Liu
Department of Chemistry, University of Pittsburgh
219 Parkman Avenue, Pittsburgh, PA 15260 (USA)
E-mail: xinyuliu@pitt.edu

L. Dalponte,[+] U. Umeobika, Dr. L. Trembleau, Prof. M. Jaspars, Dr. W. Houssen
Marine Biodiscovery Centre, Department of Chemistry
University of Aberdeen, Meston Walk, Aberdeen AB24 3UE (UK)
E-mail: w.houssen@abdn.ac.uk

L. Dalponte,[+] Dr. W. Houssen
Institute of Medical Sciences, University of Aberdeen
Aberdeen AB25 2ZD (UK)

T. Galica
Institute of Microbiology AS CR, v.v.i., Center ALGATECH, Třeboň (Czech Republic)
and
University of South Bohemia, Faculty of Science
Department of Ecosystem Biology
České Budějovice (Czech Republic)

A. Bent, Prof. J. Naismith
Biomedical Sciences Research Complex, University of St Andrews
North Haugh, St Andrews, Fife KY16 9ST (UK)

Dr. E. Rizzi, Prof. G. De Bellis
Institute for Biomedical Technologies (ITB)
National Research Council (CNR)
via F.lli Cervi 93, Segrate (MI) (Italy)

Dr. W. Houssen
Pharmacognosy Department, Faculty of Pharmacy
Mansoura University, Mansoura 35516 (Egypt)

[+] These authors contributed equally to this work.

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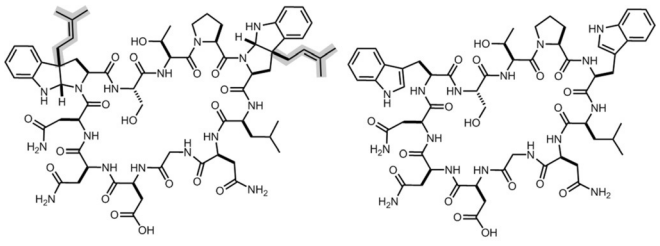

Scheme 1. The cyclic undecapeptides kawaguchipeptin A (**1**) and kawaguchipeptin B (**2**) reported from *Microcystis aeruginosa* NIES-88. Kawaguchipeptin A contains two C-3-prenylated tryptophan residues (highlighted). Prenylation of C-3 is coupled to the formation of a new ring formation as a result of bond formation between C-2 and NH in the main chain.

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 (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

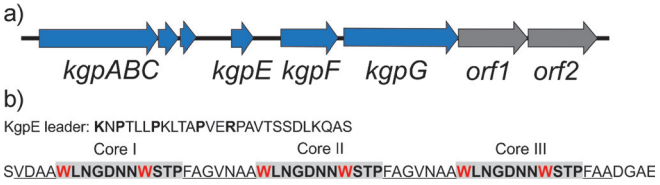

Figure 1. The kawaguchipeptin biosynthetic pathway in *Microcystis aeruginosa* NIES-88.[23] A) The 9 kb *kgp* biosynthetic gene cluster consists of six biosynthetic genes (shown in blue), organized in a single operon, that show homology to genes present in other known cyanobactin gene clusters. The grey arrows indicate genes that encode proteins with unknown functions. B) The *kgpE* precursor gene encodes the 87 amino acid precursor peptide, which contains three identical copies of the undecapeptide core (highlighted in grey).

a 9 kb gene cluster (*kgp*) together with the KgpA and KgpG cyanobactin proteases, as well as the putative KgpF prenyltransferase (Figure 1). The KgpF prenyltransferase shows just 20–45 % identity to known and putative cyanobactin prenyltransferases. A homologue of cyclodehydrase (PatD) was not detected in the gene cluster, which is consistent with the absence of heterocyclized amino acids in the cyclic peptides.

To demonstrate that the *kgp*A-G genes encode kawaguchipeptin production in vivo, we cloned the entire *kgp* operon into a broad host range yeast/bacteria shuttle vector pMQ123i[15] and placed *kgp*A downstream of a pTac promoter to generate the expression plasmid pDK-kgp1 (Figure S1 in the Supporting Information). This construct allowed the regulated expression of the *kgp*A-G genes in *E. coli* 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,

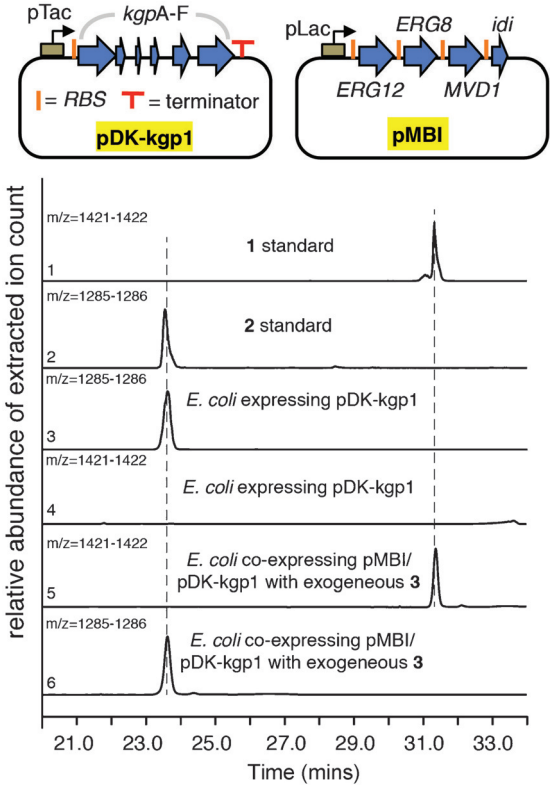

Figure 2. Heterologous expression of the *kgp* operon in *E. coli* demonstrates that the *kgp*A-G genes confer the production of **1** and **2** in vivo. Extracted ion chromatographs of LC–HRMS analysis of: Authentic **1** and **2** from *M. aeruginosa* NIES-88 (1, 2); **2** in *E. coli* transformed 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).

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,[16] 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 *kgp*A-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 1: Substrates and cofactors used for the in vitro reactions.

Substrate	Cofactor	Mono-prenylation	Di prenylation
Cyclic [WLNGDNNWSTP] (**2**)	DMAPP	+	+
Cyclic [WLNGDNNWSTP] (**2**)	IPP	+	–
Cyclic [WLNGDNNWSTP] (**2**)	GPP	–	–
Cyclic [TSQIWGSPVP] (**4**)	DMAPP	+	NA
Cyclic [SAQWQNFGVP] (**5**)	DMAPP	+	NA
Cyclic [HAFIGYDQDPTGKYP] (**6**)	DMAPP	–	–
Cyclic [RERFVYP] (**7**)	DMAPP	–	–
Cyclic [LIGIMHP] (**8**)	DMAPP	–	–
WLNGDNNWSTP (**9**)	DMAPP	+	–
WLNGDNNWSTPAYDG (**10**)	DMAPP	+	–
EDWYFDHPAYDG (**11**)	DMAPP	–	–
VPWPFPAYDG (**12**)	DMAPP	–	–
Boc-Trp (**13**)	DMAPP	–	–
Boc-Trp (**13**)	IPP	–	–
Boc-Tyr (**14**)	DMAPP	–	–
Boc-Tyr (**14**)	IPP	–	–

[+] 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, as seen in the processing of one Trp residue out of two in kawaguchipeptin B (**2**) when IPP instead of DMAPP was used (Table 1; Figures S8). We did not detect any processing of other residues in the linear or macrocyclic peptide substrates. The enzyme did not process Boc-Trp (**13**) in presence of DMAPP or IPP. 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 aestuaramides have been reported.[12] However, the latter is the result of reverse O-prenylation on the oxygen atom of Tyr followed by Claisen rearrangement.[12] The biochemical characterization of C-3 Trp prenylation[13] 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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