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Brief Communication

# Nonribosomal Peptide Synthesis in Animals: The Cyclodipeptide Synthase of *Nematostella*

Jérôme Seguin,[1,3] Mireille Moutiez,[1,3] Yan Li,[1] Pascal Belin,[1] Alain Lecoq,[1] Matthieu Fonvielle,[1,4] Jean-Baptiste Charbonnier,[1] Jean-Luc Pernodet,[2] and Muriel Gondry[1,*]
[1]CEA, Institut de Biologie et Technologies de Saclay, F-91191 Gif-sur-Yvette, France
[2]Université Paris-Sud 11, CNRS, UMR8621, Institut de Génétique et Microbiologie, F-91405 Orsay, France
[3]These authors contributed equally to this work
[4]Present address: UMR S 872, Centre de Recherche des Cordeliers, INSERM/UPMC/UPD, F-75270 Paris, France
*Correspondence: muriel.gondry@cea.fr
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## SUMMARY

**Cyclodipeptide synthases (CDPSs) are small enzymes structurally related to class-I aminoacyl-tRNA synthetases (aaRSs). They divert aminoacylated tRNAs from their canonical role in ribosomal protein synthesis, for cyclodipeptide formation. All the CDPSs experimentally characterized to date are bacterial. We show here that a predicted CDPS from the sea anemone *Nematostella vectensis* is an active CDPS catalyzing the formation of various cyclodipeptides, preferentially containing tryptophan. Our findings demonstrate that eukaryotes encode active CDPSs and suggest that all CDPSs have a similar aminoacyl-tRNA synthetase-like architecture and ping-pong mechanism. They also raise questions about the biological roles of the cyclodipeptides produced in bacteria and eukaryotes.**

## INTRODUCTION

Cyclodipeptide synthases (CDPSs) form a family of small enzymes using two aminoacyl-tRNAs (aa-tRNAs) as substrates for formation of the two peptide bonds of cyclodipeptides (Gondry et al., 2009). These enzymes are promiscuous, but have different substrate specificities. Indeed, AlbC from *Streptomyces noursei* mostly produces cyclo(L-Phe-L-Leu) (cFL), Rv2275 from *Mycobacterium tuberculosis* mostly produces cyclo(L-Tyr-L-Tyr) (cYY), and the other six experimentally characterized CDPSs mostly produce cyclo(L-Leu-L-Leu) (cLL) (Gondry et al., 2009; Lautru et al., 2002). The cyclodipeptides produced are modified by the cyclodipeptide-tailoring enzymes (Belin et al., 2009; Gondry et al., 2001) associated with CDPSs, in diketopiperazine (DKP) biosynthesis pathways. The biological role of the final DKPs in the organisms producing them remains unknown, but albonoursin, which is derived from cFL in *S. noursei,* has antibacterial activity (Fukushima et al., 1973), whereas mycocyclosin derived from cYY may be essential for *M. tuberculosis* viability (Belin et al., 2009; McLean et al., 2008; Vetting et al., 2010), and pulcherriminic acid derived from cLL chelates iron in *Bacillus* species (Cryle et al., 2010; Uffen and Canale-Parola, 1972).

CDPSs share a common ping-pong mechanism, involving a covalent intermediate in which an amino acid is transferred from an aa-tRNA to an active site serine, and a common architecture (Bonnefond et al., 2011; Sauguet et al., 2011; Vetting et al., 2010). Their structure resembles that of the catalytic domain of class-Ic aminoacyl-tRNA synthetases (aaRSs), the enzymes catalyzing the activation of amino acids and their transfer to cognate tRNAs to form aa-tRNAs. They contain a highly conserved deep pocket, which accommodates the aminoacyl moiety of the aa-tRNA substrate, and a patch of basic residues that may interact with the tRNA moiety of the substrate, as suggested for AlbC (Sauguet et al., 2011).

New putative CDPSs have recently been identified by iterative PSI-BLAST searches (Aravind et al., 2010) in various bacteria, but also in eukaryotes, such as the filamentous fungus *Gibberella*, the annelid worm *Platynereis* and the sea anemone *Nematostella*. We show here that a putative CDPS identified in *Nematostella vectensis* is an active CDPS, using a catalytic mechanism similar to that of bacterial CDPSs. We also demonstrate that all the most abundant cyclodipeptides synthesized by this new CDPS contain L-tryptophan, suggesting that new CDPSs with different substrate specificities remain to be discovered.

## RESULTS

### Putative CDPSs Found in *Nematostella vectensis*

Eight bacterial members of the CDPS family have been experimentally characterized to date (Gondry et al., 2009). However, several other putative members of this family have been detected in various bacterial phyla, and even in eukaryotes (Aravind et al., 2010). In the sea anemone *N. vectensis*, three proteins (XP_001636125, XP_001636126, XP_001621211), which we have named *Nvec*-CDPS1-3, respectively, have been detected. These proteins are encoded by three genes containing large introns (see Figure S1 available online). Comparison of the sequences of *Nvec*-CDPS1-3 showed that their C-terminal parts are almost identical and are aligned with the sequences of known CDPSs (Figure 1). The main difference between the three proteins of *N. vectensis* lies at the N-terminal part of the

 

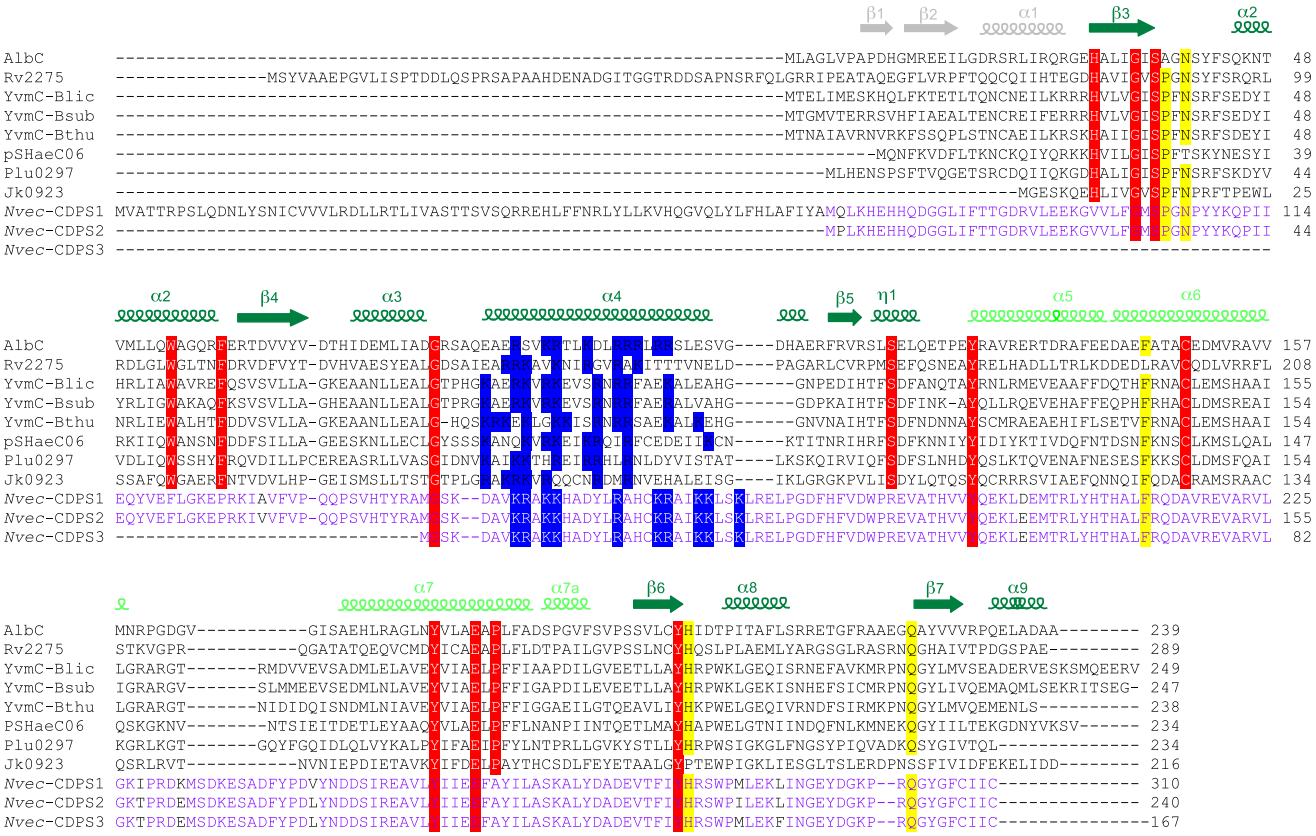


**Figure 1. Alignment of the Protein Sequences of the Eight Known CDPSs and the Three Hypothetical Proteins from *N. vectensis***

Positions with identical residues in known CDPSs are indicated by a red background. Positions with identical residues in at least 10 proteins (nine proteins if the position does not exist in the truncated *Nvec*-CDPS3) are indicated by a yellow background. Positions with identical residues in *Nvec*-CDPS1, *Nvec*-CDPS2, and *Nvec*-CDPS3 are shown in purple. The basic residues, which define a patch of positive charge, are indicated by a blue background. The secondary structural elements of AlbC (Sauguet et al., 2011) are indicated above the alignment.

See also Figure S1.

sequences, which differ in length. The *Nvec*-CDPS2 (240 residues) is of a similar size to the eight characterized CDPSs (216–249 residues in general, with only one CDPS having 289 residues), whereas *Nvec*-CDPS1 was longer (310 residues) and *Nvec*-CDPS3 smaller (167 residues). The three proteins displayed only moderate sequence similarity to the known CDPSs. For example, *Nvec*-CDPS2 displayed only 16%–21% identity and 30%–40% similarity to the known CDPSs. However, 7 of the 13 residues conserved among bacterial CDPSs are also found in *Nvec*-CDPS1 and *Nvec*-CDPS2. These residues (G35, S37, G79, Y128, Y178, E182, Y202, AlbC numbering) were previously shown to be involved in CDPS activity. In particular, residue S37 has been identified as the catalytic residue involved in formation of the covalent aminoacyl-enzyme intermediate (Sauguet et al., 2011). The hypothetical protein *Nvec*-CDPS3 appears to be truncated, because its N-terminal sequence starts ~40 residues after the essential G35 and S37 residues (Figure 1).

We generated a homology-based structural model for *Nvec*-CDPS2 with Modeler software (Sali and Blundell, 1993), using the crystal structure of AlbC (Protein Data Bank [PDB] id: 3OQV) as a template (Figure 2; Figure S2). Superimposition of the three-dimensional structures of AlbC and *Nvec*-CDPS2 gave an root-mean-square deviation value of 0.48 Å over 193 Cα. We compared the pocket in *Nvec*-CDPS2 model (Figure 2A) with its equivalent in AlbC, which accommodates the aminoacyl moiety of the aa-tRNA substrate and contains the catalytic residues (Figure 2B). The catalytic residues in *Nvec*-CDPS2, including the catalytic serine residue in particular, are located at positions compatible with their involvement in catalysis. The buried surfaces of the two pockets consist mostly of side chains of hydrophobic residues, but with a higher proportion of aromatic residues for *Nvec*-CDPS2 (Figures 2A and 2B). We also compared the distribution of charged residues on the surfaces of the two proteins (Figures 2C and 2D). The known CDPSs were found to contain a patch of basic residues thought to interact with the tRNA moiety of an aa-tRNA substrate (Sauguet et al., 2011). A similar patch is also present in *Nvec*-CDPS2 (Figure 2C), and probably also in *Nvec*-CDPS1 and *Nvec*-CDPS3 (Figure 1), based on sequence alignment.

The presence of both these basic patches and the conservation of similar residues in the three proteins strongly suggest that these proteins may be members of the CDPS family, with *Nvec*-CDPS3 being a truncated CDPS.

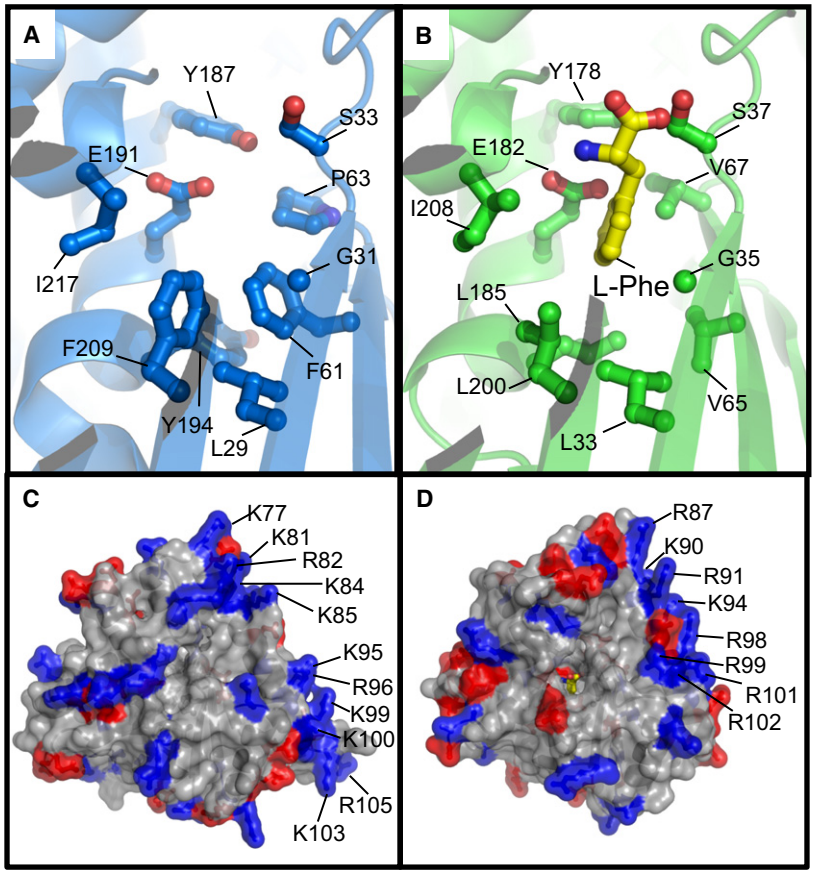


**Figure 2. Comparison of the *Nvec*-CDPS2 model with the crystal structure of AlbC**

Surface-accessible pockets of (A) *Nvec*-CDPS2 and (B) AlbC with the phenylalanyl moiety of a Phe-tRNA$^{Phe}$. Distribution of Arg/Lys (blue) and Asp/Glu (red) residues on the surface of (C) *Nvec*-CDPS2 and (D) AlbC. See also Figure S2.

## Biosynthesis of Cyclodipeptides by *Nvec*-CDPS2

We investigated the ability of *Nvec*-CDPS1 and *Nvec*-CDPS2 to catalyze the formation of cyclodipeptides. As we had previously shown that all known CDPSs, when produced in *Escherichia coli*, synthesize cyclodipeptides that are then released into the medium (Gondry et al., 2009), we introduced constructs encoding each of the two putative CDPSs separately in this host. *Nvec*-CDPS2 was produced successfully, with a proportion of this protein found in the soluble protein extract, whereas no *Nvec*-CDPS1 production was detected (Figure S3). We then analyzed the cyclodipeptide content of the culture supernatants of *E. coli* cells producing *Nvec*-CDPS2. This analysis was carried out by LC-MS/MS, without prior assumptions about the nature of cyclized amino acids, as previously reported (Gondry et al., 2009). Comparisons of the UV chromatograms for *Nvec*-CDPS2 samples and the control (culture supernatant of *E. coli* containing the empty vector) showed several peaks specific to *Nvec*-CDPS2-producing cells (Figure 3A). An analysis of the corresponding extracted ion current (EIC) chromatograms and MS/MS fragmentation patterns led to the identification of 13 cyclodipeptides (cWF, cWL, cWM, cWA, cWG, cFF, cFL, cFM, cFA, cYL, cYF, cYM, and cLA; Figures 3B and 3C). Most were combinations of an L-Trp, L-Phe or L-Tyr residue with a nonpolar amino acid (L-Leu, or L-Met or L-Ala or Gly). The three cyclodipeptides, cWF, cWL, and cWM, were the major products generated by *Nvec*-CDPS2. They were present at concentrations of 0.8–1 mg $l^{-1}$ of culture supernatant after 44 hr (Figure S4). All other cyclodipeptides were produced at concentrations of 0.1–0.5 mg $l^{-1}$ of culture supernatant, except for cFL and cWG, which were identified from retention times and fragmentation patterns but could not be quantified.

Thus, *Nvec*-CDPS2 was active in *E. coli* and the most abundant cyclodipeptides synthesized by *Nvec*-CDPS2 contained an L-Trp. This is consistent with the presence of large numbers of aromatic residues in the catalytic pocket of our structural model (Figure 2A). This is the first cyclodipeptide-synthesizing enzyme to be shown to display such specificity. Indeed, AlbC from *S. noursei*, Rv2275 from *M. tuberculosis* and the other six CDPSs generate principally L-Phe-, L-Tyr-, and L-Leu-containing cyclodipeptides, respectively (Gondry et al., 2009).

## *Nvec*-CDPS2, a New Member of the CDPS Family

We then investigated the possible use, by *Nvec*-CDPS2, of aa-tRNAs as substrates for the synthesis of cyclodipeptides. We produced *Nvec*-CDPS2 in *E. coli* and purified the protein to homogeneity by a three-step chromatographic procedure, as previously described (Gondry et al., 2009). *Nvec*-CDPS2 appears to be a monomer (Figure S5), as reported for AlbC from *S. noursei* (Sauguet et al., 2011) and YvmC from *Bacillus licheniformis* and *Bacillus subtilis* (Bonnefond et al., 2011). We then assessed the cyclodipeptide-synthesizing activity of the purified enzyme. *Nvec*-CDPS2 preferentially synthesizes Trp-containing cyclodipeptides, but it also produces significant amounts of other cyclodipeptides, including cFF (Figure 3), in vivo. We used an in vitro assay based on cFF detection, which was developed in our laboratory for AlbC, to check whether the purified *Nvec*-CDPS2 generated cFF. The enzyme-dependent formation of cFF was unambiguously detected (Figure 4). Indeed, the amount of cFF increased over time in the presence of 1 μM *Nvec*-CDPS2 (Figures 4A, 4C, and 4D), and increased with the concentration of *Nvec*-CDPS2 after 3 hr of reaction (Figure 4B). As we used *E. coli* tRNA$^{Phe}$ to assess the activity of the purified *Nvec*-CDPS2, accurate kinetic analyses would be meaningless. However, our experiments clearly show that aa-tRNAs are the substrates of *Nvec*-CDPS2, as for previously characterized CDPSs.

We then investigated whether *Nvec*-CDPS2 used a similar catalytic mechanism to bacterial CDPSs. It has been shown that bacterial CDPSs use a ping-pong mechanism and that the first step of their catalytic cycle involves a nucleophilic attack of the catalytic serine on a aminoacyl-tRNA, to form a covalent

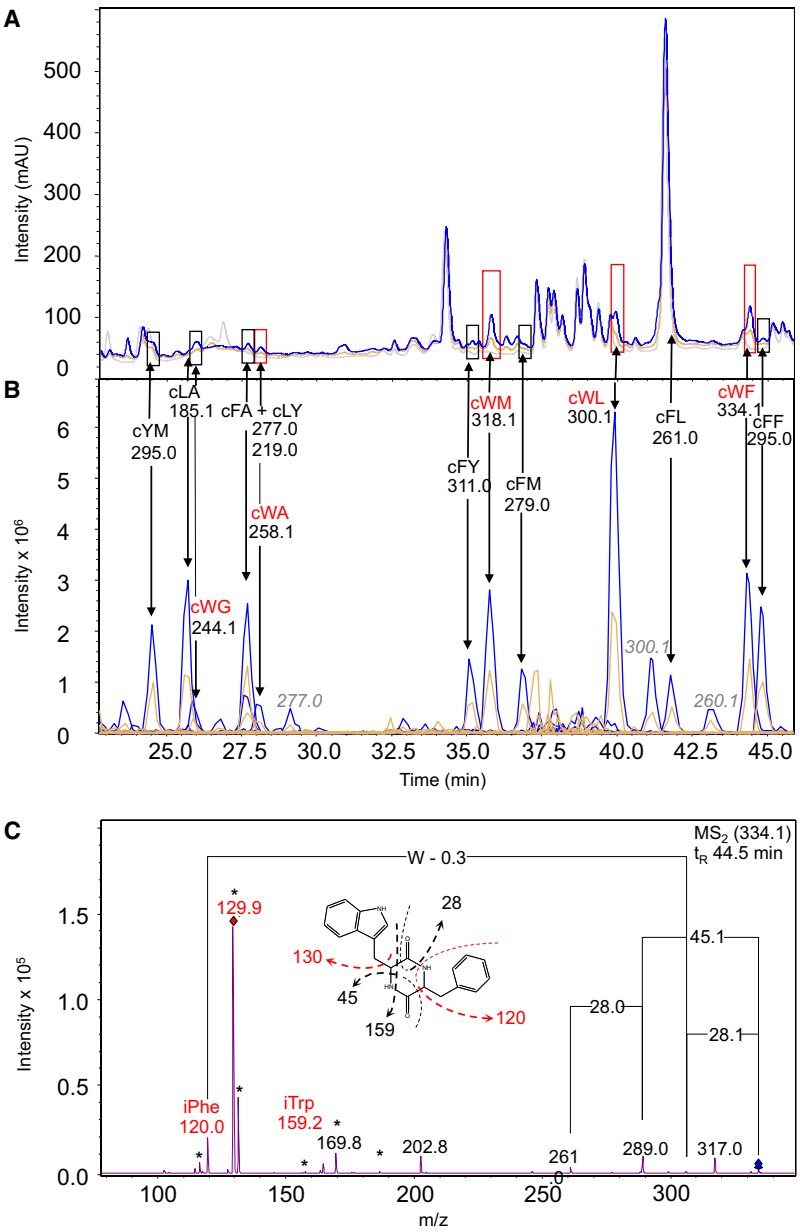


**Figure 3. LC-MS/MS Analysis of the Cyclodipeptides Secreted into the Culture Supernatant of *E. coli* Cells Producing *Nvec*-CDPS2**

(A) UV traces (λ = 214 nm) of the culture medium of *Nvec*-CDPS2-producing *E. coli* cells after 20 hr (orange) and 44 hr (blue) of expression, and of cells containing the empty vector (gray). Cyclodipeptide peaks are surrounded by a rectangle.

(B) Corresponding EIC peaks, showing the cyclodipeptide peaks and their identity.

(C) MS/MS of one of the major compounds, identified as cWF. The fragmentation pattern is characteristic of a cyclodipeptide and red labels at 120.0 ± 0.1 and 159 ± 0.1 match the immonium ions of Phe (iPhe) and Trp (iTrp), respectively. The m/z peaks marked with an asterisk are specific to Trp fragmentation (Falick et al., 1993; Papayannopoulos, 1995).

See also Figures S3 and S4.

acyl-enzyme intermediate (Vetting et al., 2010; Sauguet et al., 2011; Bonnefond et al., 2011). As *Nvec*-CDPS2 can produce cFF, this suggests that it can recognize Phe-tRNA$^{Phe}$ as a substrate. We therefore incubated *Nvec*-CDPS2 with substoichiometric amounts of [$^{3}$H]Phe-tRNA$^{Phe}$, as previously described for AlbC. Positive and negative controls were performed with AlbC and its inactive variant S37A, respectively (Sauguet et al., 2011). The proteins were separated by SDS-PAGE, transferred to a PVDF membrane and analyzed with a radioimager (Figure 4E). Radioactivity associated with *Nvec*-CDPS2 is clearly visible, indicating the formation of a covalently bound phenylalanyl-intermediate. The intensity of the signal is lower than that for AlbC, but the specificity of the two enzymes should be taken into account: AlbC mostly synthesizes Phe-containing cyclodipeptides, whereas cFF is a minor product of *Nvec*-CDPS2 activity. Our results support the hypothesis that *Nvec*-CDPS2, like its bacterial counterparts, uses a ping-pong mechanism involving a covalent aminoacyl-enzyme intermediate.

## DISCUSSION

The starlet sea anemone *N. vectensis* belongs to the phylum Cnidaria. *In silico* analysis of the draft genome of this emerging Cnidaria model (Putnam et al., 2007) led to the identification of three proteins presenting sequence similarities to bacterial CDPSs. We show here that *Nvec*-CDPS2 is a member of the CDPS family. *Nvec*-CDPS1 is probably a functional CDPS but we were unable to produce it in our experimental conditions. With the sequence data available, *Nvec*-CDPS3 appears to be a truncated CDPS, lacking essential residues.

*Nvec*-CDPS2 differs in specificity from other characterized CDPSs as it produces mostly L-Trp-containing cyclodipeptides, whereas the others synthesize L-Phe-, L-Tyr-, and L-Leu-containing cyclodipeptides (Gondry et al., 2009). The study of other putative CDPSs would extend the range of substrates known to be used by these enzymes and increase our understanding of CDPS specificity.

Cyclodipeptides are synthesized by CDPSs or nonribosomal peptide synthetases (NRPSs) (Gondry et al., 2009). NRPSs are widespread in bacteria and fungi but have not been identified so far in plants or animals. CDPSs are present in bacteria and putative CDPSs have been identified in eukaryotes, such as the fungus *Gibberella zeae* and the annelid *Platynereis dumerilii* (Aravind et al., 2010). *Nvec*-CDPS2 from *N. vectensis* is the first enzyme from animal origin experimentally demonstrated to be involved in nonribosomal peptide synthesis.

The cyclodipeptides synthesized by the known bacterial CDPSs are precursors of more complex DKPs generated by tailoring reactions (Belin et al., 2009; Gondry et al., 2001). In bacteria, the genes encoding the CDPSs and the associated

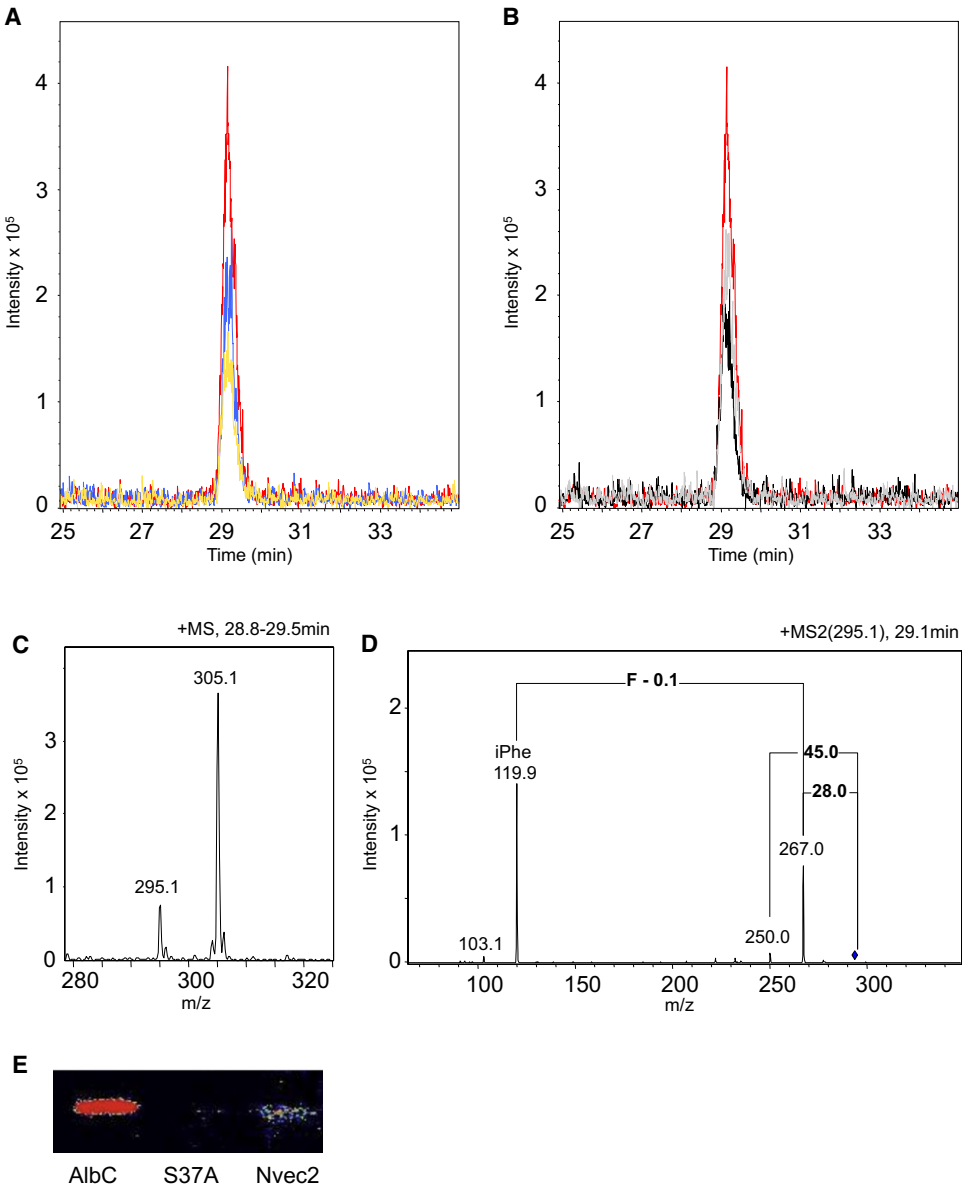


**Figure 4. In Vitro Enzymatic Assays with Purified *Nvec*-CDPS2**

(A) EIC chromatograms corresponding to the formation of cFF (m/z = 295) catalyzed by 1 μM *Nvec*-CDPS2 at 30 min (yellow), 1 hr (blue), and 3 hr (red).

(B) EIC chromatograms corresponding to the formation of cFF (m/z = 295) at 3 hr catalyzed by 125 nM *Nvec*-CDPS2 (black), 500 nM *Nvec*-CDPS2 (gray), and 1 μM *Nvec*-CDPS2 (red).

(C) Formation of cFF catalyzed by 1 μM *Nvec*-CDPS2, after 3 hr: MS spectrum of products eluted at 29.1 min, identified as cFF (m/z = 295.1) and $^{13}C_9$,$^{15}N$-labeled cFF (m/z = 305.1).

(D) MS/MS spectrum of the product formed by *Nvec*-CDPS2, identified as cFF (m/z = 295.1).

(E) Covalent labeling of *Nvec*-CDPS2 by tritiated Phe transferred from [$^3$H]Phe-tRNA$^{Phe}$, as described in Experimental Procedures. Wild-type AlbC and the S37A variant are used as positive and negative controls, respectively.

See also Figure S5.

cyclodipeptide-tailoring enzymes are clustered, facilitating the identification of tailoring enzymes and, thus, of the final DKPs. No such genetic organization is observed in the *N. vectensis* genome, so it remains unknown whether the cyclodipeptides are the final DKPs.

The physiological role of the final DKPs remains unclear. It has been suggested that DKPs act as small diffusible molecules involved in cell-to-cell communication. In bacteria, they may constitute a new class of quorum-sensing signals (Degrassi et al., 2002; Holden et al., 1999; Park et al., 2006), or even interspecies signals (Li et al., 2011). Furthermore, as DKPs have bioactive effects on their plant or animal hosts, a role in transkingdom signaling has also been suggested (Ortiz-Castro et al., 2011; Prasad, 1995). Moreover, expression of the CDPS gene in

*P. dumerilii* increases in response to septic injury (Altincicek and Vilcinskas, 2007), suggesting a possible role of the CDPS in *Platynereis* immunity (Aravind et al., 2010). The nature and role of the final DKPs remain unknown for *N. vectensis*.

## SIGNIFICANCE

**Cyclodipeptide synthases (CDPSs) form a family of small enzymes structurally similar to class-I aminoacyl-tRNA synthetases (aaRSs). They use aminoacyl-tRNAs (aa-tRNAs) as substrates to catalyze the formation of various cyclodipeptides, which are the precursors of many natural products exhibiting noteworthy biological activities. So far, all characterized CDPSs originated from bacteria. We show here that a predicted protein identified in the sea anemone *N. vectensis*, namely *Nvec*-CDPS2, belongs to the CDPS family. *Nvec*-CDPS2 is the first enzyme involved in nonribosomal peptide synthesis to be identified in animals. This finding raises questions about the biological roles of the cyclodipeptides produced in eukaryotes. *Nvec*-CDPS2, like its bacterial counterparts, exhibits an aminoacyl-tRNA synthetase-like architecture and uses a ping-pong mechanism involving a covalent aminoacyl-enzyme intermediate. However, *Nvec*-CDPS2 differs in specificity from other characterized CDPSs as it produces mostly L-Trp-containing cyclodipeptides, whereas the others synthesize L-Phe-, L-Tyr-, and L-Leu-containing cyclodipeptides. This finding suggests that new CDPSs with different substrate specificities remain to be discovered. The characterization of new CDPSs opens the way to CDPS engineering to further increase the natural diversity of cyclodipeptides, a family of compounds with diverse biological properties.**

## EXPERIMENTAL PROCEDURES

### Structural Model of *Nvec*-CDPS2

Alignments of protein sequences were obtained with MUSCLE and HHPred (Söding et al., 2005) and used for model building with Modeler (Sali and Blundell, 1993). The crystal structure of AlbC at 1.9 Å resolution (PDB id: 3OQV) was used as a template for this homology modeling. The quality of the *Nvec* model was checked with the Qmean server (Benkert et al., 2009) (Figure S2).

### Cloning, Production, and Purification of *N. vectensis Nvec*-CDPS1 and *Nvec*-CDPS2

Synthetic genes encoding *Nvec*-CDPS1 and *Nvec*-CDPS2 were purchased from GENEART and inserted into the pQE60 vector (QIAGEN), as previously described (Gondry et al., 2009). The $His_6$-tagged *Nvec*-CDPS2 protein was then produced and purified as described by Braud et al. (2005) and Gondry et al. (2009).

### Analysis of Cyclodipeptide Synthesis by *Nvec*-CDPS2

In vivo assays for *Nvec*-CDPS2 activity were performed as described by (Gondry et al., 2009). Cyclodipeptides were detected and identified from both their m/z value (MS) and their daughter ion spectra (MS/MS), as a result of their common fragmentation patterns. The nature of the detected cyclodipeptides was unambiguously confirmed by comparison with authentic standards.

### Detection of *Nvec*-CDPS2 Activity

The cFF-forming activity of *Nvec*-CDPS2 was measured by a coupled assay, as described by (Sauguet et al., 2011). Reactions were initiated by adding *Nvec*-CDPS2. Aliquots were withdrawn at various times and mixed with $^{13}C_9$,$^{15}N$-labeled cFF solution as a stable isotope internal standard, before cFF analysis by LC-MS.

### Detection of the Covalent Aminoacyl-Enzyme Intermediate

Purified *Nvec*-CDPS2, wild-type AlbC (positive control) and the AlbC variant S37A (negative control) were incubated with [$^3$H]Phe-tRNA$^{Phe}$. The tritiated substrate was obtained as previously described (Sauguet et al., 2011). The enzyme was added at a final concentration of 16 μM for wild-type AlbC or 80 μM for the variant S37A and *Nvec*-CDPS2. After 30 s of incubation for AlbC wild-type and 120 s for *Nvec*-CDPS2 and S37A, the reaction was quenched and analyzed (Sauguet et al., 2011).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.chembiol.2011.09.010.

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