New Cyt-like δ-endotoxins from *Dickeya dadantii*: structure and aphicidal activity

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In the track of new biopesticides, four genes namely cytA, cytB, cytC and cytD encoding proteins homologous to *Bacillus thuringiensis* (Bt) Cyt toxins have been identified in the plant pathogenic bacteria *Dickeya dadantii* genome. Here we show that three Cyt-like δ-endotoxins from *D. dadantii* (CytA, CytB and CytC) are toxic to the pathogen of the pea aphid *Acrithosiphon pisum* in terms of both mortality and growth rate. The phylogenetic analysis of the comprehensive set of Cyt toxins available in genomic databases shows that the whole family is of limited taxonomic occurrence, though in quite diverse microbial taxa. From a structure-function perspective the 3D structure of CytC and its backbone dynamics in solution have been determined by NMR. CytC adopts a cytolysin fold, structurally classified as a Cyt2-like protein. Moreover, the identification of a putative lipid binding pocket in CytC structure, which has been probably determined by NMR, shows that the whole family is of limited taxonomic occurrence, though in quite diverse microbial taxa.

In the track of such biopesticides, bacterial toxins did provide paradigmatic solutions, such as the crystal toxins encoded in plasmids of the soil bacterium *Bacillus thuringiensis* (Bt), which provided a vast diversity of Cry-like toxins for plant protection. Recently however, Bt-related toxins were found in genomes outside its original Gram+ bacterium: four genes namely cytA, cytB, cytC and cytD, encoding proteins homologous to *Bacillus thuringiensis* (Bt) Cyt toxins have been identified in the *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) genome. The low GC content of these genes in *D. dadantii* suggests horizontal transfer from a GC-poor *Gram*+ bacterium. Bt Cyt toxins are produced in parasporal crystals during sporulation, together with the better-known Cry toxins. So far three families of Cyt proteins, Cyt1, Cyt2 and Cyt3 have been identified (http://www.btomenclature.info). They are active by ingestion and, after proteolytic maturation, they perforate the membrane of insect intestinal cells through a presumed receptor-independent pathway, by attaching non-specifically to phospholipids. The mechanism of action of Bt Cyt toxins is not fully understood yet. The plant
pathogenic bacteria *D. dadantii* was also shown to be a pathogen of the pea aphid *Acyrthosiphon pisum* [17]. Artificial infection of *A. pismum* by *D. dadantii*, via an oral route, provokes the death of the insect in about four days. When delivered *per os*, the reduced virulence of a *D. dadantii* strain deleted for all four cyt genes suggests that these proteins are involved in its pathogenicity to insect. When the mutant is delivered by injection into the hemocoel, the virulence is not reduced, evoking an intestinal cell target for the *Dickeya* Cyt proteins [17].

To better understand the role of *D. dadantii* Cyt toxins in its pathogenicity to insect, we defined the following strategy for the present study: i) the four recombinant proteins were purified and used for toxicity bioassays against the pea aphid *A. pismum*; ii) a phylogenetic analysis was performed to investigate the evolutionary and functional relationships within the whole Cyt-like protein family; iii) from a structure-function perspective, the CytC 3D structure and its dynamics in solution have been determined by NMR.

This integrative approach provided significant insights into the evolutionary history of *D. dadantii* Cyt toxins, which appear to be interesting leads for biopesticides, in parallel to the screens still performed within the *Bacillus thuringiensis* species.

**Results**

**Protein purification and protein compliance.** To investigate the biological activity of the four *D. dadantii* Cyt proteins, we tried to produce these proteins as GST-fusion proteins expressed in *E. coli* cells and purify them. Some difficulties were encountered in producing some of these proteins in our bacterial system due to their low solubility. We could not produce CytD protein because it was highly insoluble and formed inclusion bodies. Low production levels were obtained for CytA and CytB (less than 40 µg for 1 L of culture). The quantity purified was however sufficient to perform some biological tests. The production level of the CytC protein was far better (around 0.4 mg for 1 L of bacterial culture). SDS-PAGE analyses showed that the CytA, CytB, CytC proteins were of high purity, and with the expected size (Figure S1). Due to its production level, CytC was selected for further NMR structural studies. The purified protein recovery was independent of isotopic feeding conditions.

**Biological assays.** Different biological assays were performed to assess, and compare the functionalities of the produced proteins with those of the *B. thuringiensis* proteins.

**Insect bioassays.** Figure 1 summarize the insect toxicity bioassays performed by ingestion with the pea aphid and four available Cyt-like proteins: one from *Bacillus thuringiensis* subs. *israelensis*, a typical solubilized dipteran-specific toxin Cyt1A, and the three available *D. dadantii* toxins (CytA, CytB and CytC).

Survival analysis of the associated data showed that all tested proteins induced weak but significant acute mortality on pea aphid nymphs between 250 and 1000 µg/mL, with the following quantitative trends: Cyt1A (UniProt ID: P0A383) intoxication at 1000 (resp. 250 µg/mL) resulted in LT50 (Lethal Time 50%, in days, and confidence interval under a Weibull fit) of 3.24 [2.11–4.98] and 12.8 [10.3–16.0] respectively. In the parallel assay, *D. dadantii* CytC (UniProt ID: E0SJ33) at 1000 (resp. 250 µg/mL) resulted in LT50 of 10.1 [5.9–17.3] and 9.6 [8.4–11.0] respectively. In the same assay at 500 (resp. 250 µg/mL), Cyt B (UniProt ID: E0SJ34) resulted in LT50 of 5.1 [3.7–7.1] and 8.1 [7.3–9.1] respectively. Due to toxin availability and stability issues with the recombinant CytB and CytA proteins (not CytC), assays were not all performed with the same concentration range or in a single bioassay batch. In an independent experiment, CytA (UniProt ID: E0SJ35) resulted in LT50 of 2.28 [1.84–2.82], 15.7 [8–31] and 22 [11–43] respectively at 1000, 500 and 125 µg/mL.

Overall, the different bioassays showed that all three tested Cyt toxins displayed aphical activities somewhat similar to that of Cyt1A [18], with a strong growth-reducing effects (Figure 1) in the 250–500 µg/mL range. Comparing all experiments resulted in a global ranking of toxicity as follows: *Dda*-CytB > *Dda*-CytC ≈ *Bth*-Cyt1A ≈ *Dla*-CytA. More precisely, LT50 of Cyt B is the smallest at active dose (e.g. 8.1 days at 250 µg/mL). The most striking phenotypical effect, previously observed with *B. thuringiensis* Cyt1A [18] as a severe growth impairment of the pea aphid at moderate doses, was reproduced with *D. dadantii* Cyt endotoxins with more than 60% growth inhibition, CytB being the most active at low active dose (Figure 1).

**Hemolysis assays.** No hemolytic activity was observed for the CytA (up to 120 µg/mL, 5.4 µM), CytB (up to 30 µg/mL, 1.3 µM) and CytC (up to 1000 µg/mL, 45 µM) proteins. This is to compare to the low nanomolar range (44 nM) published for the hemolytic activity of Cyt1A [18], reproduced in our assays as a full hemolysis for trypsin-activated Cyt1A toxin at less than 1 µM. Trypsin did not activate CytC in our hemolysis assays.

**Phylogeny of the Cyt-like family.** We aimed at identifying the whole set of proteins homologous to the *D. dadantii* and *B. thuringiensis* Cyt proteins. The Blast-based homology search on UniProt and Genbank peptide databases retrieved 70 non-redundant sequences, which were aligned and analyzed through a maximum-likelihood phylogeny, presented as an unrooted tree in Figure 2 (450 patterns out of 715 sites, 277 non-polymorphic sites -39%-). Deep branches identified the three independent clades that were only recently grouped within a single family through structural alignments [20,21]; we named these the volvatoxin clade, the Evf clade and the bacterial Cyt clade. All these proteins share the cytotoxin fold. In order to investigate the bacterial Cyt clade with more accuracy, we excluded the two other clades to reduce saturation and re-performed the analysis (Figure 3; 404 patterns out of 563 sites, 163 sites -29%- found without polymorphism). The canonical *Bacillus* (Firmicute) Cyt toxins were grouped in two well resolved clusters, the Cyt 1 and Cyt 2/3 clades; the only taxonomical outlier in the latter cluster was the *Streptomyces* (Actinobacteria) toxin. The remaining clusters were
Figure 2 | Unrooted phylogenetic tree of all non-redundant cyt-homologous toxins that were retrieved from Genbank, Uniprot and specific genome databases. Phylogenetic reconstruction, on aligned protein sequences followed a PhyML method\textsuperscript{50} with a WAG 4-rate class model. The early three separate clades are labeled evf, volvatoxin and (other) bacterial cyt clades. Labels are built to allow both easy reading and non-ambiguous peptide identification: Bacillus thuringiensis toxins are not preceded by the species suffix (otherwise: three letter suffix-id, Gsp for Genus species), follow the Crickmore holotype classification, and labels include the Uniprot accession number for non-Bt species.
**Figure 3** Phylogenetic tree of all bacterial cyt toxins; tree is unrooted but tentative rooting at longest branch is proposed. Method and labels as in figure 2.
**Cyt toxin family nomenclature.** Figures 2 and 3 represent to our knowledge the most comprehensive Cyt trees to-date, and contain phylogeny-based nomenclatural updates for the Cyt toxin family, as defined and classified previously. We propose that, independent on gene namings, proteins are short-named “only” Cyt[A-Z] and described as « cytolytic delta-endotoxin » (as most-often on gene namings, proteins are short-named “only” « Cyt[A-Z] » and described as « type-[N] cytolytic delta-endotoxin » (as most-often encountered in Uniprot and exemplified by accession P0A382 for example). When clusters of paralogs are found in single genomes, such as in *Dickeya* sp., the [A–Z] suffix should ideally be attributed only after adequate orthology assignment is performed within the given clade.

**CytC solution Structure.** CytC structure was determined by NMR on the protein labeled with $^{15}$N and $^{13}$C. Chemical shift assignments were obtained for 95% of the backbone and 75% of the proton side-chains (BMRB code 19834). The NMR 3D structure of CytC (PDB entry 2MLW) was determined using NOE distances, dihedral angles and hydrogen bonds (Table 1). As a member of the Cyt protein family, CytC has a cytolysin fold, i.e. a single domain of $\alpha/\beta$ architecture consisting of a $\beta$-sheet surrounded by two $\alpha$-helical layers (Figure 4). The sheet consists of 4 main anti-parallel $\beta$-strands having a modified Greek key topology composed of $\beta_2$ (T137-G149), $\beta_3$ (G153-T166) and $\beta_4$ (L182-V193) connected by a longer link to $\beta_1$ (A63-K74), which is adjacent to the first strand $\beta_2$. The sheet is flanked by two $\alpha$-helical layers: $\alpha_1$ (Q29H39) and $\alpha_2$ (K51-A58) on one side, and $\alpha_3$ (L79-E93) and $\alpha_4$ (N106-F112) on the other (Figure 4). Some of the NMR models contain a supplementary very short $\beta$-strand (F124-N127), antiparallel to $\beta_2$.

Figure 4 | Ribbon representation of model 1 (A) and 11 (B) of the NMR ensemble of CytC (2MLW.pdb). Overlay of $\alpha$ traces of models 1 to 10 (C) and 11 to 20 (D). (E) Topology diagram of CytC. Helices and strands are represented by circles and triangles respectively. Secondary structure elements are colored in rainbow starting from blue to red.

| Table 1 | NMR constraints and structural statistics |
|----------|-------------------------------------------|
| **NMR constraints** | |
| Distance restraints | |
| Total NOE | 4522 |
| Unambiguous | 4276 |
| Ambiguous | 246 |
| Hydrogen bonds | 83 |
| Dihedral Angle Restraint | 351 |
| Structural Statistics (2 mlw.pdb) | |
| Models 1–10 | Models 11–20 | |
| Average violations per structure | |
| NOEs $\geq$ 0.3 Å | 0 | 0 |
| Hydrogen bonds $\geq$ 0.5 Å | 1 | 1 |
| Dihedrals $\geq 10^\circ$ | 0 | 0 |
| Average pairwise rmsd (Å) | 0.357 | 0.329 |
| | 0.467 | |

Ramachandran Analysis

| Most favored region | 60.4% |
| Allowed region | 34.2% |
| Generously allowed | 4.0% |
| Disallowed | 1.4% |

not resolved basally, which resulted in the unsolved positioning of the *Clostridium* (Firmicute) group as related to the *Dickeya*, *Aeromonas* groups (Proteobacteria). The *D. dadantii* toxins derive from lateral gene transfers as testified by their still-evolving GC% within large genomic pathogenicity clusters. The recovery of the same 4-cyt gene toxin cluster in the eight *Dickeya* genomes shows that this linkage predates the speciation events within this bacterial group. Within the Cyt toxins sensu stricto (i.e. excluding Efv and Volvatoxin clades), the most probable scenario seems to be an invention/diversification of the toxins within the Firmicutes (*Bacillus/Clostridium*) followed by three episodes of horizontal gene transfers towards independent and distant bacterial clades (*Dickeya*, *Aeromonas*, *Streptomycetes*).
0.91 ± 0.12 s⁻¹ respectively. However, residues A128 to V136, comprised between β2 (T137-G149) and β3 (G153-T166) exhibit lower values for the ¹⁵N heteronuclear NOE and transverse relaxation rate than the rest of the protein indicating enhanced mobility.

For rigid protein molecules, in the limit of slow molecular motion (τc ≫ 0.5 ns) and high magnetic field, a closed-form solution for τc, as a function of the ratio of the longitudinal (T1) and transverse (T2) ¹⁵N relaxation times exists:

\[ \tau_c \approx \frac{1}{4\nu_N} \sqrt{\frac{T_1}{T_2} - 7} \]  

(1)

where νN is the ¹⁵N resonance frequency (in Hz). This equation 1 is derived from Eq. 8 from Kay et al.²⁴ by considering only J(0) and J(ν0) spectral density terms and neglecting higher frequency terms. Using this equation, CytC τc was estimated to be 13 ns. By comparing this to a table of rotational correlation time values for known proteins, we can conclude that our NMR sample of CytC has a molecular weight around 22 kDa and that it is a monomer.

**Discussion**

The core of this study was to investigate the role in pathogenesis of the homologous Cyt-proteins that are present in *D. dadantii* genome. Consequently, we focused on the following key question: do CytA, CytB, CytC and Cyt D proteins present insecticidal activities? To address this question, toxicity bioassays against the pea aphid were performed with recombinant form of the protein expressed in *E. coli*.

The biological activity data clearly show that the *Dda*-Cyt genes encode insecticidal proteins active against the pea aphid, and that this activity was at least as potent as that of its parent *Bth*-Cyt1A protein.²⁴ Protein CytD could not be produced in the expression system used. However, its persistent outward positioning and long-branching in the phylogeny may reflect a structural peculiarity or a nascent pseudogenisation process. The activity range of CytA, CytB branching in the phylogeny may reflect a structural peculiarity or a intoxication in an aphid 25, consistent with the membrane-related cytocytes was shown to be the most visible cellular phenotype of Cyt2A toxin action. Impairment of the microvillar structure of aphid enterocytes being three to four times smaller than normal. This is a clear severe growth-stunting effect (Figure 1), resulting in surviving individuals being three to four times smaller than normal. This is a clear indication of lack of physiological adaptation of the gut cell to the toxin action. Impairment of the micrerval structure of aphid enterocytes was shown to be the most visible cellular phenotype of Cyt2A intoxication in an aphid²⁵, consistent with the membrane-related mode of action of Cyt toxins, either through a detergent or a pore-forming mechanism.²⁵

Noteworthy, no synergism between Cyt1A and any of the tested Cry toxins was observed with the pea aphid (Porcar and Rahbé, unpublished), in contrast to the situation described with Cry11 on mosquito²⁶²⁷. All these results indicated that the Cyt toxins of *Dickeya dadantii* were probably evolved outside their original *Bt* ecosystem to perform (alone) their pore-forming function in the digestive tract of target insects. This is consistent with the previous finding of i) a virulence function when the bacteria was ingested but not by injection, as measured by differential virulence of *wt* and Δ*cyt D. dadantii strains*²⁸, and ii) gut-restricted expression of the cyt operon.²⁸ One series of interesting and selective features of the *Dda*-Cyt toxins is i) their lack of hemolytic activity, as compared to the parent *Bt*-Cyt toxins²⁹,³⁰ ii) their N-terminal shortening, and hence absence of need for proteolytic activation, which was experimentally checked in the present work for hemolytic activity, and iii) their ability to be expressed alone in a bacterial cytoplasm, whereas the *Bt*-Cyt toxins sometimes display bacterial cell toxicity³⁰ and need a helper gene to be expressed in a standard intracellular bacterial context.²⁸ An analysis of Cyt1Aa mutants has shown that the mutations V122E and V126E affected strongly the oligomerization and haemolytic properties of the proteins.²⁹ Interestingly, the homologous residues in CytC, the least prone to aggregation of the *D. dadantii* toxins, are Q89 and E93, which could explain the properties of CytC. However, other changes should explain the absence of haemolytic activity of CytA and B since a leucine is found at the position corresponding to V122 and a valine is conserved at the position corresponding to V126 (Figure 5). It seems unlikely to us that the distinctive properties of the *D. dadantii* Cyt properties could have evolved without appropriate selective pressures. The first trait (hemolysis) should involve target organism specificity, while the other trait (bacterial compatibility) should involve adaptation to recipient host cytoplasmic expression. Both are meaningful but deserve further experimental analysis.

The phylogenetic analysis of the comprehensive set of Cyt toxins available in genomic databases shows that the whole family is of limited taxonomic occurrence, though in quite diverse microbial taxa. Together with some information on their genomic context (e.g. positioning in pathogenicity islands and GC content, for the *Dickeya dadantii* toxins), this is indicative of a diversification in a somehow restricted and specialized set of species (e.g. Firmicutes/ *Bacillus* species), followed by rare recurring events of horizontal gene transfers (HGT) and fixation in other lineages (such as in some fungal –*Volvariella, Gibberella*– or γ-proteobacterial –*Dicyea, Aeromonas*– species). A possible positioning of a root in the presented tree was attempted for such a scenario (Figure 3): the longest identified branch between the Cyt2 clade and the other *Bacillus* taxa could be this basal point, leading to a set of three successive events of HGT into unrelated taxa (*Streptomycyes, Aeromonas* and *Dicyea*). *Clostridium* is a much closer taxon to *Bacillus*, and its positioning in the tree is less clear. It is interesting to note that a related group of filamentous bacteria, *Arthromitus* (Firmicutes, Clostridiales), have long been described in association to arthropods and insects, and showed both fossil and extant records of association with insects.³⁵

Whether a new set of toxin folds, modules and assemblages (both the Cyt and Cry toxins contain specific pore-forming folds) arose from such a position in the bacterial tree may be seen as a challenging hypothesis, not properly studied yet even in the most comprehensive genomic analyses of the *Bacillus* sp genomes published to date, which show that both cry and cyt genes occur exclusively on plasmids in the 45 *Bacillus* genomes explored.³⁴ At the other end of the tree, the reconstructed topology (Figure 3) shows clearly that the *Dicyea* toxin cluster evolved in a multistep process involving a double tandem-duplication step, giving birth to the CytBC clade (proteins shorter than the two other groups, ~200 residues vs ~220 residues for CytA and CytD).

From a structure-function perspective, the tridimensional structure of the *Dicyea* CytC toxin, and its backbone dynamics, were determined by NMR spectroscopy. CytC NMR structure is very similar to the crystal structures of the mature monomer Cyt1Aa (3RON.pdb)³⁹, of the endogenously cleaved Cyt2Ba monomer
and the corresponding region of Cyt2Aa (1CBY.pdb) despite their low sequence identity (25%; Figures 6 and 7). Unlike Bt Cyt toxins, the Dickeya CytC was not cleaved to obtain a soluble toxic monomer, and this ability is confirmed by a correlation time $t_c$ indicative of a monomeric state. Cyt1Aa contains an insertion of a $b$-hairpin between $a_1$ and $a_2$ which is common to all members of the Cyt1 family and is absent from the Cyt2 family and from the Cyt proteins of D. dadantii. We can then conclude that CytC is structurally a Cyt2-like protein. This is confirmed by a higher percentage of structural similarity between Cyt2Aa, Cyt2Ba and the “closed” con-

(2RCI.pdb) and the corresponding region of Cyt2Aa (1CBY.pdb) despite their low sequence identity (~25%; Figures 6 and 7). Unlike Bt Cyt toxins, the Dickeya CytC was not cleaved to obtain a soluble toxic monomer, and this ability is confirmed by a correlation time $t_c$ indicative of a monomeric state. Cyt1Aa contains an insertion of a $b$-hairpin between $a_1$ and $a_2$ which is common to all members of the Cyt1 family and is absent from the Cyt2 family and from the Cyt proteins of D. dadantii. We can then conclude that CytC is structurally a Cyt2-like protein. This is confirmed by a higher percentage of structural similarity between Cyt2Aa, Cyt2Ba and the “closed” con-
The formation of CytC, 65% than between Cyt1Aa and CytC, 59–60% (Figure 7). The most accepted mechanism by which Cyt proteins damage cell membrane is similar to the one proposed for the volvatoxin A2 36,37 (1VCY.pdb). Firstly, the protein undergoes conformational changes where the two outer α-helical layers swing away from the β-sheet. The β-sheet is then able to bind on the cell membrane and finally oligomerization on the cell membrane forms β-barrel pores. Nevertheless, recent studies suggested that oligomerisation is a prior step before Cyt1Aa membrane insertion. The NMR structure of CytC is clearly in agreement with the first step of this proposed mechanism as α-helices could have the ability to swing away from the β-sheet in solution. Moreover, this behavior could explain the lack of stability of the protein. Indeed, once in “open” conformation, oligomerization might occur, leading to protein precipitation. The pores, in Cyt1Aa, have been proposed to be formed by three major β-strands (β6–β8) which are structurally conserved in CytC (β2–β4) 38.

CytC, like other Cyt family members, also has a fold similar to that of the virulence factor Evf (2W3Y.pdb) despite its very low (~15%) sequence identity. In the case of Evf, a palmitate covalently bound by a cysteine is found in a hydrophobic pocket embedded between the β-sheet composed of β3, β5, β6 and β7 strands and α4 and α5 helices (Evf numbering). The structural homology between Cyt proteins and Evf enabled the identification of a putative fatty acid binding site in all Cyt1 and Cyt2 protein between the sheet formed by β4, β6–β8, and helices α3–α5 (Cyt1Aa numbering) 38. CytC NMR structures also exhibit a quite large hydrophobic pocket defined between the β-sheet and α3 and α4 in the “closed” conformation (models 1 to 10) and of course absent in the “opened” conformation (models 11 to 20) (Figure 5). Moreover the hydrophobic residues delineating the cavity are conserved among the Cyt family members (Figure 6). The presence of this hydrophobic cavity constitute a strong evidence that the common ancestor of Evf and the cytolytic toxins contained a lipid binding site which has been maintained in the two clades, and probably in most members of the Cyt-toxin family, since Eca-Evf, Bth-Cyt2B and Dda-CytC are located at diverse positions of the phylogenetic tree (Figure 2). These data support the importance of this putative lipid-binding cavity for the mechanism of action of Cyt.
and Cyt-like family members, since hydrophobic interactions would clearly prevail at the membrane binding site.

In conclusion, the susceptibility of aphids to *D. dadantii* Cyt endotoxins, and some specificities of the latter such as their non hemolytic properties, may lead to the development of effective strategies for controlling such sucking pests with genetically modified crops expressing the toxins. However, two conditions should concur:

(i) Toxins must be expressed in the plant phloem to be accessible to these pests and (ii) more effective toxins should be found or engineered. As example, Chougule and collaborators have improved toxicity of *Bacillus thuringiensis* toxin Cyt2Aa against hemipteran insect pests. Insertion of a 12-amino-acid pea aphid gut-binding peptide by adding to or replacing amino acids in one of three loops of the *Bt* cytolytic toxin, Cyt2Aa, has resulted in enhanced binding and toxicity against both the pea aphid, *Acrithosiphon pisum*, and the green peach aphid, *Myzus persicae*. The exploration of our results may end up in a new protein family lead for the control of aphids and related insect pests, which include some of the most important pests of global agriculture.

**Methods**

Over-expression of *D. dadantii* cytA and cytB and *^1^H* and *^1^C* labeling of CytC, cytA was amplified with the oligonucleotides cytA + (5'-gccgcaggggccacagtactgagcggc-3') and CytA- (5'-gcgctgggtgtcatgctgggtggtcgcggc-3'). cytB was amplified with the oligonucleotides CytB + (5'-cggctgtgctcatgtcggggtctgtgctgcgtc-3') and CytB- (5'-gcgctgggtgtcatgctgggtggtcgcggc-3'). cytC was amplified with the oligonucleotides CytC + (5'-cggctgtgctcatgtcggggtctgtgctgcgtc-3') and CytC- (5'-gcgctgggtgtcatgctgggtggtcgcggc-3'). and cytD was amplified with the oligonucleotides CytD + (5'-cggctgtgctcatgtcggggtctgtgctgcgtc-3') and CytD- (gcgctgggtgtcatgctgggtggtcgcggc-3'). The amplified DNA were digested with BamHI and Xhol and ligated into pGEX-6p3 plasmid (GE Healthcare) digested with the same enzymes. The pGEX derivatives producing the fusion GST-Cyt proteins were introduced into *E. coli* NM522 strain. Cells were grown in LB medium to OD600 0.8 and induced with 1 mM isopropylthiogalactoside (IPTG) for 3 h.

Labeling of CytC was performed according a method adapted from Marley et al. *E. coli* NM522/pGEX-CytC was grown in 1 L of LB medium. When OD600 reached 0.8, bacteria were collected by centrifugation and resuspended in 1 L of M63 medium containing *^1^H* and *^1^C*. After 1 h, 10 mL of a 200 mM glucose solution and 1 mM IPTG were added. Cells were grown overnight and treated as described thereafter.

Purification of recombinant labeled and unlabeled proteins, and toxin activation. Cells were collected by centrifugation, resuspended in buffer A (50 mM, Tris pH 7.0, 100 mM NaCl, 1 mM EDTA) and frozen in a French cell press. Unbroken cells were collected by centrifugation. GST-Cyt proteins were bound on Protino Glutathione Agarose 4B (Macherey-Nagel) equilibrated with buffer A, washed several times with the same buffer and the Cyt proteins were liberated by addition of Prescission protease (GE Healthcare) according to the manufacturer's protocol. The proteins used for bioassay tests were dialyzed extensively against pure water and freeze-dried.

For checking the potential effect of proteolytic cleavage on bioactivity, preliminary hemolysis and insect bioassays with activated/non activated CytC were performed. Purified (desalted, lyophilised) CytC was activated by trypsin (Sigma P7926) directly in AP3 pH 7.5 insect diet. Toxin was incubated at 500 mg/mL with purified trypsin at 1/25 toxin/protease ratio, for 16 h at 25°C. Protoplasts were performed on a 600 MHz Varian INOVA spectrometer at 298K. Spectra were measured with NMRPipe46 and analysed with CCPNMR (version 2.1.5)47. Backbone and side-chain resonance assignments were obtained from the standard triple resonance experiments48. Interproton distances were derived from 3D *^1^H*-HSQC-NOESY and *^1^H*-HSQC-NOESY datasets obtained at a mixing time of 100 ms. Backbone dihedral angle restraints were determined with DANGLE programs49.

Structures were calculated with NOE distances, hydrogen bonds and θ and ψ angles using ARBIA (version 2.3)21. The ARBIA protocol used simulated annealing with torsion angle and Cartesian space dynamics with the default parameters, including water refinement of the structures. The iterative process was repeated until the convergence of the NOE spectra was complete. The last run was performed with 500 initial structures out of which 20 were selected on the basis of total energies and restraint violation statistics, to represent the structure of CytC in solution. The figures were prepared with PYMOL. *^1^H* NMR and *^1^C* NMR spectra were acquired with 32 scans per point, with a recycle delay of 3.0 s. Relaxation delays of 10, 50, 100, 200, 300, 400, 500, 600, 700, 800, 1000, 1500, 2000 and 3000 ms were used for data collection. Relaxation delays of 10, 30, 50, 70, 90, 110, 130, 150, 170, 190, 210, 230, 250, 310, 350 and 410 ms were used for data collection. *R*1 and *R*2 were obtained using a single exponential decay function.

The *^1^H*-NOE spectra were collected with a 3 s presaturation period and a 3 s relaxation delay; the reference experiment had an equivalent 6 s delay. The *^1^H*-H*^1^N* heteronuclear NOE was calculated from the equation NOE = *I* sat/*I* eq with *I* sat and *I* eq the intensities of a cross peak in the spectra collected with and without presaturation respectively.

**Sequence alignment for structural analyses.** Sequence alignments were performed using CLUSTAL OMEGA23 and analyzed with JalView24 programs.

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Author contributions
K.C. carried out the NMR spectroscopy and structure calculation and dynamics analyses. D.C. performed and analysed the insect assays. G.E. carried out the over-expression of four D. dadantii cyt proteins and 13C labeling of CytC. G.C. participated in the over-expression of four D. dadantii cyt proteins and 13C labeling of CytC and carried out the hemolysis assay. Y.R. performed the phylogeny analyses and participated in the insect assays. C.L. participated and analysed the sequence alignment for structural analyses. P.D.S. conceived of the study and participated in its design and coordination and drafted the manuscript.

Additional information
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