**INTRODUCTION**

Inhibitor cystine-knots (ICKs) form a family of ultra-stable miniproteins, found in a wide variety of organisms, with confirmed and potential medical applications. They are characterized by the presence of at least three interwoven disulfide bridges, which form an intramolecular knot and confer them structural and functional resistance to high temperature, enzymatic degradation, extreme pH and mechanical stress. ICKs are ∼30–50 residue long, which make them easily accessible to chemical synthesis. The loops connecting the disulfide bridges show a high variability of sequence, which results in a broad range of functions covered by ICKs, from channel blockage to inhibition of enzymes. All these properties have lead to use ICKs as scaffolds for the engineering of various pharmaceuticals; functions and evolution. The website also provides access to bibliographic data and to computational tools that have been specifically developed for ICKs. Here, we present a major upgrade of our database, both in terms of data content and user interface. In addition to the new features, this article describes how KNOTTIN has seen its size multiplied over the past ten years (since its last publication), notably with the recent inclusion of predicted ICKs structures. Finally, we report how our web resource has proved usefulness for the researchers working on ICKs, and how the new version of the KNOTTIN website will continue to serve this active community.

**ABSTRACT**

Knottins, or inhibitor cystine knots (ICKs), are ultra-stable miniproteins with multiple applications in drug design and medical imaging. These widespread and functionally diverse proteins are characterized by the presence of three interwoven disulfide bridges in their structure, which form a unique pseudoknot. Since 2004, the KNOTTIN database (www.dsimb.inserm.fr/KNOTTIN) has been gathering standardized information about knottin sequences, structures, functions and evolution. The website also provides access to bibliographic data and to computational tools that have been specifically developed for ICKs. Here, we present a major upgrade of our database, both in terms of data content and user interface. In addition to the new features, this article describes how KNOTTIN has seen its size multiplied over the past ten years (since its last publication), notably with the recent inclusion of predicted ICKs structures. Finally, we report how our web resource has proved usefulness for the researchers working on ICKs, and how the new version of the KNOTTIN website will continue to serve this active community.

**KNOTTIN: the database of inhibitor cystine knot scaffold after 10 years, toward a systematic structure modeling**

Guillaume Postic¹,²,³,⁴, Jérôme Gracy⁵, Charlotte Périn¹,²,³,⁴, Laurent Chiche⁵ and Jean-Christophe Gelly¹,²,³,⁴,*

¹INSERM, U 1134, DSIMB, 6, rue Alexandre Cabanel, 75739, Paris Cedex 15, France, ²Université Paris Diderot, Sorbonne Paris Cité, UMR_S 1134, 75739 Paris, France, ³Institut National de la Transfusion Sanguine, 75739 Paris, France, ⁴Laboratory of Excellence GR-Ex, 75739 Paris, France and ⁵CNRS UMR 5048, INSERM U1054, Centre de Biochimie Structurale, Université Montpellier, 34090 Montpellier, France

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Specific tools

Besides the database, the KNOTTIN website is also a platform that regroups under the ‘Tools’ menu softwares dedicated to the analysis of knottin proteins. These computational methods are Knoter1D and Knoter3D (7), which are aimed at identifying knottins based on their 1D sequences and 3D structures, respectively. The third section of the ‘Tools’ menu is a portal to our Knoter1D3D web server (8) for the prediction of knottin 3D structures based on their sequence. The KNOTTIN web site also provides access to statistics about the database content, citations and web traffic. General information about knottins and the database usage are also available for users. Finally, the ‘Links’ menu contains hyperlinks to knottin-related web resources—such as the Cyclotide Webpage (www.cyclotide.com), CyBase (www.cybase.org.au), ToxProt (www.uniprot.org/program/Toxins), MvirDB (9) and the ConoServer (10,11)—and other protein databases and web servers.

NEW FEATURES

Since its launch, the KNOTTIN database has seen its size grow (from 385 sequences in 2004, to 3320 in 2017, and from 85 to 214 natives structures), as the number of available sequences in UniProt and native structures in the PDB increased. However, the amount of experimentally determined structures of knottins remains relatively limited compared to the number of sequences, due to the difficulties related to the purification and crystallography of these proteins. This lack of available structures is a critical concern regarding the knottin-based drug design, which mainly lies on the study of 3D structures. To overcome this issue, one of the main features of this upgraded version of KNOTTIN is the systematic and automatic modeling and inclusion in the database of theoretical models produced with our aforementioned Knoter1D3D tool. This addition of predicted structures for every knottin sequence has increased by one order of magnitude the size of our database in terms of

Querying the system

The database can also be searched. Under the ‘Sequence search’ menu, amino acids sequences can be searched with the BLAST algorithm. The theoretical and experimental models of KNOTTIN can also be searched, under the ‘Conformation search’ menu, based on different structural features, such as torsion angles, secondary structure content and solvent accessibility. Sequences and structures can also be accessed via the ‘Information search’ menu, which offers users the possibility of searching the database by using different criteria, such as family, keywords, crystallography techniques or the aforementioned knottins nomenclature. The database also gathers the literature about the knottin proteins, which can be accessed under the ‘Article search’ menu based on criteria, such as articles authors, publication date and keywords. Bibliographic data about knottins functions, folding, synthesis, modeling, and biotechnological applications can also be browsed under the different sections of the ‘Bibliography’ menu.

PRACTICAL USE

Over the past decade, KNOTTIN has been cited by multiple articles and reviews. In most of the cases, our database is cited either as a source of information on knottins, or when introducing knottin proteins and their structural characteristics, various functions, or their presence in a wide range of species. Numerous citations of KNOTTIN are also related to the families of knottins defined in the database, to which authors refer when identifying or quantifying knottins of interest. This shows that our database, throughout the years, has successfully served as a useful overview on the field of inhibitor cystine knots. In addition to the data stored in KNOTTIN, the computational tools available on the website have also been cited, in particular Knoter1D and Knoter3D (for example in (12–14)). Our database has also been cited by three patents, including one describing cystine knot peptides engineered for anti-thrombotic therapies (15) and which suggests using the KNOTTIN’s conformation search to determine folding patterns.

DESCRIPTION

Website navigation

The content of the KNOTTIN database can be browsed with the horizontal navigation bar of the web interface. The ‘Experimental 3D structures’ and ‘Sequences & 3D models’ menus give access to experimental and theoretical models, respectively. In these pages, users can select one or several proteins, to visualize either their aligned sequences or 3D structures. The latter can also be done under the ‘Sequence alignments’ menu, which displays pre-compiled multi-sequence alignment files. In each of these three menus, the knottins are grouped by families, which have been determined based on sequence similarity and biological activity. Each knottin of the database is also categorized based on the length of its loops between the knot-forming cysteines (i.e. five loops for the knottins, and six for the cyclotides). Thus, a sequence (a)b.c(d)[e] is attributed to each knottin, the letters ‘a’ to ‘e’ being the loops lengths. This nomenclature of ICKs has been introduced with the initial release of the database in 2004.

drophobic core formed by disulfide bonds, require adapted 2D and 3D representation. The whole diversity of knottins functions (such as neurotransmitters, analgesics, anti-infective agents, protease inhibitors, toxins and insecticides) also has to be properly represented and documented, by gathering relevant functional annotations and bibliographic data. Finally, the active community of researchers working on the applied and theoretical aspects of knottins needs easy access to softwares dedicated to the analysis of knottins, which is provided by the computational tools available on our KNOTTIN website. Here, we present an upgraded version of KNOTTIN (www.dsimb.inserm.fr/KNOTTIN/), 10 years after its last publication (7). This report describes the new features of the database, the way it has evolved and shown usefulness over the past decade, and future developments.
Figure 1. Flowchart describing how the KNOTTIN database is generated. The Knoter1D and Knoter3D processes have been defined in the previous release of KNOTTIN (7); the Knoter1D3D process is also described in our previous work (8). The UniProt data are automatically extracted, by using a Perl script, from the corresponding UniProt web pages.

structures, with currently contains >3000 theoretical models. In details, these theoretical models constitute a valuable source of structural data, especially for knottin families for which there is no experimental structure available, such as the bacterial knottins.

The prediction of knottin structures has been integrated as a step in the pipeline for generating the data of KNOTTIN (Figure 1). When a protein sequence from UniProt is identified as a knottin by Knoter1D, it is used as an input for Knoter1D3D to produce theoretical models, which will be added to the database—along with related UniProt data (such as, sequence, descriptor, species, tissue, authors, PMID, keywords) and a multi-sequence alignment with other knottins of the same family computed with ClustalW (16). The rest of the KNOTTIN pipeline concerns the detection and the subsequent addition of native knottin structures to the database. Thus, when a knottin structure is identified in the PDB by Knoter3D, the coordinate file is ‘standardized’: each residue is renumbered so that the knotted cysteines (except Cys IV) correspond to the positions 20, 40, 60, 80 and 100; the coordinate file is also reoriented by superimposition of the knotted cysteines onto those of the structure of the squash seed trypsin inhibitor (PDB code: 2btcI). These coordinate files are added to the database, along with data regarding structural properties (such as torsion angles, secondary structures, solvent accessibility) computed with STRIDE (17) and PDBgeo (described in (18)).

It should be noted that predicted structures of knottins can also be found in other databases of protein theoretical models (such as SWISS-MODEL (19) and ModBase (20)), but these data do not match the models from KNOTTIN, neither quantitatively nor qualitatively. Indeed, according to the Protein Model Portal (www.proteinmodelportal.org), which contains models from SWISS-MODEL and ModBase, there are only 468 predicted structures of knottins in these two databases. The much greater number of models in KNOTTIN is explained by the fact that our Knoter1D3D comparative modeling procedure can accurately predict knottin structures when the template-to-sequence identity is as low as 10% (8), which is rather common among knottins. Moreover, our use of
Figure 2. Visualization of the structural superimposition of three native structures of knottins belonging to the ‘Agouti-related’ family (PDB codes: 1hykA, 1mr0A and 1y7jA). A right click on the JSmol viewer allows users to modify the representations of structures, or to perform other actions.

The current version of the KNOTTIN database now contains more than 3000 sequences of knottins, and has greatly extended its reach with the addition of predicted structures for all of these sequences. To cope with the daily increase of the number of sequence in the UniProt database, future efforts will be put in the full automation of the update pipeline.

CONCLUSIONS AND PERSPECTIVES

This new version of the KNOTTIN website is distinct from the former by the updated content of its database, as well as by its new interface and the inclusion of new data types. The KNOTTIN database now contains more than 3000 sequences of knottins, and has greatly extended its reach with the addition of predicted structures for all of these sequences. To cope with the daily increase of the number of sequence in the UniProt database, future efforts will be put in the full automation of the update pipeline. Regarding the latest data, it is interesting to observed that, while
sequences have been found in animals, plants, fungi, bacteria and viruses, knottins are still absent from archaea, which converges with previous findings (13). Therefore, particular attention will be paid to new data about these organisms, which represent one of the three domains of life. Finally, KNOTTIN® is also a web server providing a user-friendly access to our knottin-specific tools. Following this direction, the platform will integrate the future computational methods we will develop for the analysis of knottin proteins.

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