Functional and structural characterization of PII-like protein CutA does not support involvement in heavy metal tolerance and hints at a small-molecule carrying/signaling role

Khaled A. Selim1,2, Lorena Tremiño2, Clara Marco-Marín3, Vikram Alva2, Javier Espinosa4, Asunción Contreras4, Marcus D. Hartmann2, Karl Forchhammer1 and Vicente Rubio3

1 Interfaculty Institute for Microbiology and Infection Medicine, Organismic Interactions Department, Tübingen University, Germany
2 Department of Protein Evolution, Max Planck Institute for Developmental Biology, Tübingen, Germany
3 Instituto de Biomedicina de Valencia (IBV-CSIC), CIBER de Enfermedades Raras (CIBERER-ISCIII), Valencia, Spain
4 Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, Spain

The PII-like protein CutA is annotated as being involved in Cu2+ tolerance, based on analysis of Escherichia coli mutants. However, the precise cellular function of CutA remains unclear. Our bioinformatic analysis reveals that CutA proteins are universally distributed across all domains of life. Based on sequence-based clustering, we chose representative cyanobacterial CutA proteins for physiological, biochemical, and structural characterization and examined their involvement in heavy metal tolerance, by generating CutA mutants in filamentous Nostoc sp. and unicellular Synechococcus elongatus. However, we were unable to find any involvement of cyanobacterial CutA in metal tolerance under various conditions. This prompted us to re-examine experimentally the role of CutA in protecting E. coli from Cu2+. Since we found no effect on copper tolerance, we conclude that CutA plays a different role that is not involved in metal protection. We resolved high-resolution CutA structures from Nostoc and S. elongatus. Similarly to their counterpart from E. coli and to canonical PII proteins, cyanobacterial CutA proteins are trimeric in solution and in crystal structure; however, no binding affinity for small signaling molecules or for Cu2+ could be detected. The clefts between the CutA subunits, corresponding to the binding pockets of PII proteins, are formed by conserved aromatic and charged residues, suggesting a conserved binding/signaling function for CutA. In fact, we find binding of organic Bis-Tris/MES molecules in CutA crystal structures, revealing a strong tendency of these pockets to accommodate cargo. This highlights the need to search for the potential physiological ligands and for their signaling functions upon binding to CutA.
Introduction

PII proteins are widespread and ancient signaling molecules first reported in *Escherichia coli* as nitrogen metabolism controllers (reviewed in [1]). They are characteristic homotrimers presenting a (βαβ)3 ferredoxin subunit fold (reviewed in [2,3]). Their targets are other protein molecules, either enzymes, transporters, or gene expression regulators (reviewed in [2,3]). Initially grouped into GlnK and GlnB classes depending on whether they were or were not involved in ammonia transport control [1,4], they initiated a superfamily that contains canonical PII proteins of known function as well as noncanonical members [5,6].

Despite the highly conserved structure, amino acid sequence conservation within the superfamily can be quite low [6,7]. The CutA protein family belongs to the families with the lowest sequence similarities to the canonical PII protein family. In fact, CutA was identified as a member of the PII superfamily because it was ‘fished’ in transitive BLAST searches using as baits the sequences of some Nif3 proteins (COG3323 entry in the EGGNOG database, http://eggnogdb.embl.de; they exhibit significant homology with PII [5]) and also because of the structural similarity of CutA with canonical PII [8]. However, the taxonomic distribution of CutA is even wider than that of canonical PII, which is not present in eukaryotes except Archaeplastida, suggesting an important role across phyla.

There is very little sound information on the functions of CutA proteins. Nevertheless, these proteins are widely annotated in databases as ‘Divalent-cation tolerance protein CutA’, and ‘Involved in resistance toward heavy metals’. These annotations stem from the fact that the gene encoding CutA, called cutA1, was first identified in *Escherichia coli* in investigations of a locus, cutA, involved in divergent metal tolerance [9]. The cutA locus contains three genes: one encoding a small PII-like protein of ~13 kDa (CutA1) located in one operon, and two encoding inner membrane proteins (cutA2-3). Mutation in the cutA locus caused increased sensitivity of *E. coli* cells to divalent metal ions (copper, zinc, nickel, cobalt, and cadmium). Complementation of ΔcutA locus with the ORF encoding CutA1 conferred enhanced tolerance only toward elevated Cd2+ levels, while it did not confer tolerance against Cu2+, Zn2+, or Ni2+. Only complementation with both, cutA1 and cutA2 genes, restored the tolerance toward high Cu2+ and Ni2+ levels. From these data, the product of the cutA1 gene was speculated to confer heavy metal tolerance or to be involved in divalent cation homeostasis [8,9], leading to the above-indicated annotation without further physiological examination in other bacterial species.

Because of the wide distribution of CutA proteins among species, the investigation of the functions of these proteins has considerable interest. For example, in humans, cutA mRNA (with several splicing forms [10]) and the protein itself are widely distributed through the body, with CutA protein levels being highest in the brain (https://bgee.org/?page=gene&gene_id=ENSG00000112514; https://www.proteomicsdb.org/proteomicsdb/#protein/proteinDetails/49653/expression), where CutA appeared to be involved in the processing, trafficking, and membrane-anchoring of acetylcholinesterase, although not via direct interactions with this key neurotransmitter-processing enzyme [10–13]. In addition, a membrane-anchored splicing isoform of CutA interacts with the membrane-bound β-secretase BACE1, which produces the β-amyloid (βA) neurotoxic peptide centrally linked to Alzheimer disease [14]. CutA knockdown or overexpression influenced in opposite directions βA secretion in an in vitro cellular system [14]. Indirect evidence has linked (reviewed in [15]) CutA to human reproduction and development, in particular to the growth and differentiation of embryonic stem cells, the survival and maturation of oocytes, the recognition of the gametes in the female genital apparatus, and the activation of secretion and development of the mammary gland. In fact, cutA has been identified [16] as one of the 35 key genes for milk secretion. The specific roles of CutA in all these processes are far from clear.

Although protein structures are believed to be crucial for deciphering the molecular bases of function [17], the present structural knowledge of CutA proteins does not parallel our (rather poor) understanding of the functions of these proteins. The first crystal structures of CutA proteins were determined for the bacterium *E. coli* (EcCutA; PDB: 1NAQ) and the...
mammal *Rattus norvegicus* (RnCutA; PDB: 1OSC) [8]. Both proteins formed trimers, indicating the evolutionary conservation of the trimeric architecture of PII-like proteins [8]. As already indicated, the subunits of the trimer exhibited a ferredoxin-like fold with a β1x1β2-β3x2β4 architecture. The fold included a small β-hairpin loop connecting strands β2 and β3, as well as an additional small C-terminal β5-α3 extension. In an EcCutA crystal structure, a Hg²⁺ metal ion was found bound within one of the intersubunit clefts [8], which was considered suggestive evidence for the involvement of CutA in heavy metal sensing.

To gain further insights into the distribution of CutA proteins among all domains of life, we performed a bioinformatic analysis to identify different subgroups of CutA proteins and their relation to the other members of the PII superfamily. In order to understand their function in cyanobacteria, we chose two cyanobacterial CutA proteins from *Synechococcus elongatus* PCC 7942 and *Nostoc* sp. PCC 7120 for physiological, biochemical, and structural characterization. In addition, we reexamined with up-to-date microbiological tools whether *cutA1* is or is not important for Cu²⁺ resistance of *E. coli*.

### Results

**Cluster analysis of CutA proteins reveals widespread distribution among all domains of life**

To ascertain the sequence conservation of CutA homologs across prokaryotes and eukaryotes, we carried out PSI-BLAST [18] searches seeded with representative CutA homologs. Our searches revealed that the CutA protein family is quite conserved, with most homologs sharing over 35% pairwise sequence identity; the human and *E. coli* CutA homologs, for instance, exhibit a pairwise sequence identity of ~43%. To detect other protein families homologous to CutA, we searched the PDB70 profile-HMM database using the remote homology detection method HHpred [19], with the *E. coli* CutA protein as query. While the best matches, as expected, were to CutA proteins from other organisms, we also found matches to many PII proteins at HHpred probability values of >90%, suggesting that CutA and PII proteins share a common ancestry. Consistent with our observation, they are also classified under the same homology level in the SCOPe and ECOD protein classification databases [20].

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**Fig. 1.** Cluster map of CutA proteins. CutA proteins were gathered from the nr90 database using PSI-BLAST and were clustered in CLANS [21] based on the strength of their all-against-all pairwise sequence similarities. In the map, each dot represents one protein sequence and the sequences belonging to the same taxonomic group are shown in one color. BLAST connections are shown as gray lines to indicate the significance of sequence similarities; the darker a line, the higher the significance. Bacterial sequences are colored in light red, cyanobacterial sequences in red, archaean sequences in green, and eukaryotic sequences in violet. The CutA proteins from *E. coli* (EcCutA), *Nostoc* sp. PCC 7120 (NsCutA), and *S. elongatus* PCC 7942 (SeCutA) are indicated by arrows.
To comprehensively explore the taxonomic distribution and evolutionary conservation of CutA proteins, we searched the nr90 database, a version of the nonredundant protein sequence database filtered to a maximum pairwise sequence identity of 90%, for CutA homologs using PSI-BLAST and investigated the obtained homologs using cluster analysis. The PSI-BLAST search yielded a total of 5736 homologs, which we subsequently clustered in CLANS [21] based on the statistical significance of their all-against-all pairwise sequence similarities, as measured by PSI-BLAST P-values. Since CutA proteins exhibit high pairwise sequence identities, we chose a stringent P-value cutoff to achieve separation between the archaeal, bacterial, and eukaryotic sequences (Fig. 1). At our chosen cutoff for clustering (1e-32), while the archaeal (colored green in the map) and bacterial (light red) sequences formed many loosely connected single clusters, exhibiting significant diversity, the eukaryotic (violet) sequences formed a single cluster, underpinning their high sequence conservation. The eukaryotic CutA proteins are found primarily in alveolates, kinetoplastids, metazoans, and green plants, but are largely absent in fungi. By contrast, the prokaryotic CutA proteins are distributed across almost all lineages of bacteria and archaea, including the deep-branching bacterial classes Actinobacteria and Cyanobacteria as well as the Asgard group of archaea, which is thought to represent the closest prokaryotic relatives of eukaryotes [22]. The cyanobacterial sequences (red) are mostly organized into two groups, but some are scattered across the map. While one of the two cyanobacterial groups is located near the eukaryotic cluster, the other is more distant.

To gain insights into the cellular function of CutA, in particular, the potential involvement of cyanobacterial CutA proteins in heavy metal tolerance, we chose two cyanobacterial homologs for biochemical and structural analysis. One of them, from the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 (ScCutA encoded by the ORF *Synecc7942_2261*), is located close to the eukaryotic cluster (Fig. 1), whereas the other one, from the filamentous cyanobacterium *Nostoc* sp. PCC 7120 (*Ns*CutA encoded by the ORF *alr7093*), is located at the periphery of the map (Fig. 1). We note that these proteins are the only CutA paralogs in these two organisms.

**CutA does not mediate heavy metal tolerance in cyanobacteria**

The physiological importance and possible role of CutA in the sensitivity of cyanobacteria to divalent metal cations were investigated by gene inactivation in *S. elongatus* PCC 7942 and *Nostoc* sp. PCC 7120 (from now on *S. elongatus* and *Nostoc*, respectively). First, we inactivated *S. elongatus* cutA (*Synecc7942_2261*) by allelic replacement with the kanamycin-resistant allele cutA::Tn5 from cosmid 8S34-E4 [23] (Fig. 2A). After transformation of *S. elongatus* with cosmid 8S34-E4, candidate clones (KmR CmS) were isolated and subjected to PCR analyses. Complete segregation of the inactive cutA::Tn5 alleles was easily achieved (Fig. 2B), confirming the prior finding [24] that cutA is not an essential gene in *S. elongatus*. An alternative approach also generating cutA null kanamycin-resistant mutants in *S. elongatus* based on a pUC19 plasmid derivative as previously described for *Synechocystis* sp. PCC 6803 [7] was also successful (Fig. 2C).

The sensitivity of *S. elongatus* wild-type and cutA mutant to transition metals was monitored using cells grown to mid-exponential phase, which were spotted on the surface of BG11 agar plates supplemented or not with the indicated metal cation (done with Cu2+, Zn2+ and Co2+, Fig. 2D). The addition of these metals significantly impaired cell growth, judged from the cell biomass required in the inoculum to observe growth. No differences in the sensitivities to these metals were observed between the wild-type and the cutA mutant (Fig. 2D). The number of metal cations tested was extended to also include Cr2+, Cd2+, Ni2+, Mn2+, Fe2+, and Pb2+, using growth assays in 24-well plates in liquid BG11 medium containing variable concentrations (maximal range, 0–50 μM) of these cations. Almost all tested conditions, the mutant behaved with no notable difference from the wild-type strain (not shown). The cutA mutant only showed some differential sensitivity against high concentration of Pb2+ (Fig. 2D). In nature, Pb2+ is normally one of the trace heavy metals and occurs at very low concentrations, suggesting that the observed phenotype of the cutA mutant is not relevant in a natural environment.

In the filamentous multicellular cyanobacteria *Nostoc* sp. PCC 7120, attempts to inactivate by allelic replacement of *alr7093* gene (encoding CutA) with a kanamycin resistance cassette were unsuccessful and the inactive alleles could not be completely segregated (Fig. 3A), suggesting that cutA might be essential for the filamentous lifestyle of *Nostoc*. Therefore, we resorted to a previously implemented antisense-RNA knockdown strategy [25,26] based on transformation of *Nostoc* sp. PCC 7120 with the promoter-less pAM1956 vector carrying in a reverse-complement orientation the gene to be knocked down (*alr7093*) preceded by a strong copper-inducible promoter (PpetE)
and followed by a promoter-less reporter (GFPmut2) (Fig. 3B,C). The antisense cutA-expressing Nostoc strain (AS cutA strain) showed GFP fluorescence (Fig. 3D), reflecting expression of the downstream reporter, and thus of the alr7093 antisense-RNA even at the low Cu²⁺ concentrations (around 0.3 µM) present in the growth medium (BG11 medium). However, the AS cutA strain did not show increased metal sensitivity (tested for Cu²⁺, Fe²⁺, Pb²⁺, Co²⁺, Zn²⁺, Cr²⁺, Cd²⁺, Ni²⁺, and Mn²⁺; illustrated only for Cu²⁺ in Fig. 3D)
The cells fully recovered as fast as the wild-type strain when returned to medium devoid of added metals (shown for Cu$^{2+}$ in Fig. 3E, drop plating). In addition, continuous growth monitoring in BG11 liquid medium (Fig. 3F) suggests that the AS cutA encoding plasmid might cause slight growth inhibition by itself, while no effect was observed by the addition of 25 µM Cu$^{2+}$ to the AS cutA strain, as it was also the case for the wild-type strain. Finally, microscopic examination (Fig. 3G) also did not reveal any morphological evidence for differential toxic effects between untreated and Cu$^{2+}$ (25 µM)-treated AS cutA cells, similarly to what was the case with wild-type cells. Overall, data on both cutA knockout in S. elongatus and cutA knockdown in Nostoc are consistent with CutA having little if any bearing on metal sensitivity of cyanobacteria under normal laboratory growth conditions.

CutA does not mediate tolerance to Cu$^{2+}$ in Escherichia coli

The failure to substantially increase divalent metal sensitivity of two cyanobacteria by knockout or knockdown of cutA prompted us to re-examine the role of the corresponding E. coli gene, cutA1, since it was from data on this bacterium that the annotation of a role for this gene in divalent cation protection had stemmed [9]. We used strain JW4097 of the Keio collection [27], in which the cutA1 gene of E. coli K-12 is replaced by a kanamycin resistance cassette (Fig. 4A). To enable the use of a pET15b expression plasmid carrying cutA1 for complementation studies, we first lysogenized the JW4097 cells as well as the parental wild-type strain BW25113 [27] with phage λDE3, which carries the gene for T7 RNA polymerase. This lysogenization had no effect on the metal sensitivity of the wild-type or the JW4097 mutant strains (data not shown).
The sensitivity of the JW4097 strain to Cu\textsuperscript{2+} was considerably higher than that of the parental wild-type strain, as shown in drop cultures in which a 10\textsuperscript{4}–10\textsuperscript{5}-fold higher cell inoculum was required with the JW4097 mutant than with the wild-type strain for growth in the presence of 3 mM CuSO\textsubscript{4} (Fig. 4B). However, this finding cannot be attributed conclusively to the lack of cut\textsubscript{A1}, since the initial 25 bases of the cut\textsubscript{A2} coding sequence (file P36655, UniProtKB database, https://www.uniprot.org/uniprot/P36655) overlap with the final bases of cut\textsubscript{A1} (Fig. 4A), and thus, a polar effect with lack of cut\textsubscript{A2} expression was anticipated in the mutant. Therefore, we examined separately the ability of cut\textsubscript{A1} and cut\textsubscript{A2} to complement this mutant strain in the copper sensitivity assays by transforming strain JW4097 with pET15 plasmids carrying either cut\textsubscript{A1} or cut\textsubscript{A2} (Fig. 4C). While resistance to Cu\textsuperscript{2+} was restored to wild-type level by transformation with cut\textsubscript{A2} even in the absence of IPTG (the inducer of the T7 promoter; this finding indicates promoter leakiness), cut\textsubscript{A1} did not provide any growth advantage in the presence of 3 mM Cu\textsuperscript{2+} over the same strain transformed with the empty parental plasmid (Fig. 4C). The lack of complementation by cut\textsubscript{A1} was not due to lack of cut\textsubscript{A1} expression, as proven by SDS/PAGE of cell extracts, which showed a band with the expected mass for CutA which was not present in the cells transformed with empty parental plasmid (Fig. 4D). Therefore, E. coli CutA does not provide protection against Cu\textsuperscript{2+}, indicating that CutA proteins from phylogenetically distant bacteria are not involved in resistance to heavy metal, the function previously attributed to CutA proteins.

**Structural characterization of SeCutA and NsCutA**

Although belonging to bacteria from the same taxonomic group (cyanobacteria), the CutA proteins from *S. elongatus*, SeCutA, and from *Nostoc*, NsCutA, share only 26% sequence identity, and SeCutA is 12 residues longer at its N terminus than NsCutA (Fig. 5A). In fact, the sequence of SeCutA resembles more in both length and identity (38%) the sequence of *E. coli* CutA (EcCutA) than the one of NsCutA (Fig. 5A). Thus, these two cyanobacterial CutA proteins could have important structural differences. This prompted us to explore these differences at the

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**Fig. 4.** Copper sensitivity of *E. coli* cut\textsubscript{A1} mutant and lack of complementation by transformation with cut\textsubscript{A1}-carrying plasmid. (A) cut\textsubscript{A1}-cut\textsubscript{A2} gene cluster of *E. coli* K-12 in wild-type (WT) strain BW25113, and in mutant strain JW4097, in which cut\textsubscript{A1} is replaced by a kanamycin resistance cassette. To the right, electrophoretic analysis of the products obtained from the two strains after PCR amplification of the region encompassed between oligonucleotides P1 and P2. (B,C) Drop cultures of decreasing (from top to bottom) cell concentrations of both strains (lysogenized with λDE3 phage, see Materials and Methods) on agar-LB medium lacking or containing 3 mM copper sulfate as indicated. In (C), the cells were transformed with the indicated plasmid (see method section and text). (D) SDS/PAGE analysis (15% polyacrylamide gel) of 4 × 10\textsuperscript{8} cells of *E. coli* JW4097 transformed as indicated. The gel was stained with Coomassie blue. The arrow marks the position of the CutA protein band.
structural level, while providing first examples of cyanobacterial CutA structures. With this goal, we produced SeCutA and NsCutA in E. coli from their encoding genes carried in expression vectors. SeCutA was tagged either N-terminally with a poly-His tag (this was the form used here unless indicated), or with
a C-terminal Strep tag, while NsCutA was Strep-tagged C-terminally (see Materials and Methods). These forms, purified with appropriate affinity columns, behaved as trimers in gel filtration, as shown by comparison with the elution of protein standards (Fig. 5B) or by co-elution with the canonical PII protein from S. elongatus PCC 7942 (SePII, a very well-studied trimeric PII protein [2,3]) (Fig. 5B, inset).

We obtained crystals of SeCutA, called here SeL0, SeL1, SeL2, and SeL3, under four different conditions (Table 1). They diffracted X-rays at excellent resolutions (1.17–2.0 Å) and had similar monoclinic unit cells in space group P2_1. NsCutA yielded an orthorhombic crystal form in P2_12_1 (Ns1, 1.9 Å resolution), and a trigonal one in P321 (Ns2, 2.45 Å). All structures could be solved by molecular replacement, and refinement yielded models with very good geometries (Table 1). The asymmetric units (ASUs) of the four SeCutA crystals contained identical single homotrimers (Fig. 5C; root mean square deviation (rmsd) values for superimposition of Cz atoms, 0.18–0.63 Å; mean, 0.48 Å) which hosted in each crystal a different number of bound molecules of Bis-Tris buffer (Table 1 and see below). Of note, the SeL2 crystal was grown in the presence of 0.22 mM CuSO_4 (Table 1) and yielded a model at excellent resolution (1.8 Å) that should have allowed the detection of bound Cu^{2+}, but none was observed, in agreement with the conclusion from our in vivo experiments that cyanobacterial CutA is not involved in protection against Cu^{2+}. Among the SeCutA crystals, SeL3 yielded the highest resolution (1.17 Å) structure ever reported for a CutA protein (Table S1). This crystal structure will be used with preference in further descriptions. Concerning the NsCutA crystals, the ASU of Ns1 contained two identical homotrimers forming a bottom-to-bottom dimer of trimers (Fig. 5D), while the ASU of crystal Ns2 contained six CutA monomers. Each of these monomers in Ns2 belonged to a different trimer that was built by application of the crystal symmetry and that, by application of that symmetry, showed the same hexamerization behavior as in Ns1 (not shown). All trimers from Ns1 and those generated by application of crystal symmetry in Ns2 superimpose closely, with rmsd values ≤ 0.35 Å. In the case of the SeCutA crystals, no hexamers or higher oligomers than the trimer were observed or could be generated by crystal symmetry operations. The trimers found in the ASUs of the different SeCutA crystals superimposed quite well mutually and with those of NsCutA (Fig. 5C, and...
Table 1. Crystallization, X-ray data collection, and refinement statistics for the crystals of the two cyanobacterial CutA proteins studied here. Values in parenthesis are the data for the highest resolution shell.

| Protein | SeCutA | NsCutA |
|---------|--------|--------|
| Crystal | Sel0f | Sel1 | SeL2 (Cu²⁺) | SeL3 | Ns1 | Ns2 |
| PDB file | 6GDU | 6GDV | 6GDW | 6GDX | 6T76 | 6T7E |
| Reservoir solution | 0.1 M MIBb pH 4, 25% PEG1500 | 0.1 M Bis-Tris pH 5.5, 0.2 M Li₂SO₄, 25% PEG3350 | 0.1 M Bis-Tris pH 6.5, 29% PEG3350, 0.22 mM CuSO₄ | 0.1 M Bis-Tris pH 6.5, 23% PEG3350 | 2 M (NH₄)₂SO₄, 0.1 M Bis-Tris pH 6.5 | 0.1 M MES pH 6.5, 1.2 M K/Na tartrate |
| Data collection | Synchrotron/Beamline/ Detector | ALBA/BL13/ Dectris Pilatus 6M | Diamond/I03/ PSI Pilatus 6M |
| Wavelength (Å) | 0.9794 | 0.9794 | 0.9795 |
| Space group | P2₁ | P2₁ | P2₁ |
| Unit cell parameters | a, b, c (Å) | 35.2, 96.4, 49.0 | 34.6, 96.6, 48.9 | 34.7, 95.8, 48.8 | 35.6, 96.3, 48.9 | 62.1, 90.7, 93.7 |
| α, β, γ (°) | 90, 94.2, 90 | 90, 97.4, 90 | 90, 97.1, 90 | 90, 93.2, 90 | 90, 90, 90 |
| Solvent (%) | 42 | 45 | 42 | 44 | 35 | 49 |
| Resolution range (Å) | 48.19–1.75 | 48.32–2.00 | 47.91–1.80 |
| Complete reflections (cell) | 32,752 (1,772) | 20,245 (1,498) | 28,906 (2,129) |
| Completeness (%) | 99.9 | 94.2 | 98.7 |
| Redundancy (%) | 7.2 | 5.6 | 10.7 |
| Rmerge (%) | 12 | 16 | 3.9 |
| Bond angle (°) | 1.50 | 1.35 | 1.35 |
| Number of Polypeptide chains | 3 | 3 | 3 |
| Protein atoms | 2462 | 2521 | 2447 |
| Water molecules | 122 | 135 | 131 |
| Ligand molecules | 0 | 1 Bis-Tris | 2 Bis-Tris |
| Average B-factor (Å²) | 15.5 | 25.2 | 31.6 |

The FEBS Journal 288 (2021) 1142–1162 © 2020 The Authors. The FEBS Journal published by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies. K. A. Selim et al.
rmsd values for trimer–trimer Ca-superimpositions in the range 1.20–1.37 Å; mean for 32 superimpositions, 1.27 Å), also exhibiting virtually perfect 3-fold molecular symmetry. Given the elution of NsCutA as a trimer in size-exclusion chromatography (Fig. 5B) and the low energy (7 kcal·mol⁻¹) predicted to be needed for hexamer dissociation to trimers (PISA server prediction, https://www.ebi.ac.uk/pdbe/prot_int/pistart.html), a physiological occurrence of the hexamer appears unlikely. Thus, the hexameric assembly will not be regarded further.

The SeCutA and NsCutA homotrimers (Fig. 5C, E and F) conform with the architecture of PII superfamily proteins (Fig. S1), which are homotrimers of subunits folded as a ferredoxin-like core followed by a C-terminal extension (Fig. 5G, H). In the core, the two interlocking βββ motifs form a four-strand antiparallel β-sheet, whereas the two antiparallel helices sit on the sheet’s outer surface (Fig. 5E, F). The folding of the C-terminal extension, somewhat variable among different members of the PII superfamily (Fig. S1), includes in CutA a beta strand (β5) followed by an alpha helix (α3) (Fig. 5G, H). A particular structural feature of SeCutA that has not been reported in other CutA proteins is the folding of its 12-residue N-terminal extension (the part missing in NsCutA (Fig. 5A), but present although not visible in the structure of EcCutA [8]) as an extra beta strand, called here β0, which interacts in an antiparallel manner with β5 from the same subunit, (Fig. 5I) perhaps helping stabilize the trimeric fold of SeCutA (see below).

In canonical PII proteins, the two βββ motifs of the ferredoxin fold are connected by the long, surface-exposed, T-loop (Fig. S1B). Instead, in CutA proteins including SeCutA and NsCutA, strands β2 and β3 are very long and are connected by a very short tip loop called here vestigial T-loop (Fig. 5G, I). These strands form a β hairpin that is kinked at the middle, changing its direction by nearly 90°. The distal part of this hairpin forms one edge of a central hybrid molecular antiparallel β sheet (Fig. 5I) composed of eight β strands with the topology β3β2β2β3β3β3β5β5 (β0), where the four strands of the ferredoxin fold of one subunit (in italic lettering) form the center of the sheet, the distal part of the β2β3 hairpin (marked with the superscript D) of the preceding subunit (in the view of Fig. 5E) forms one edge of the sheet, and β5β0 (underlined; β0 is in parentheses because it exists only in SeCutA) of the third subunit form the other edge of the sheet (Fig. 5I). Because of the participation of the β2β3 hairpin of each subunit in two adjacent sheets, the three sheets that nucleate the trimer become continuous (Fig. 5I). Of note, the β4 strand and the
preceding B-loop that connects it to helix α2 are shorter in CutA than in canonical PII (Fig. 5A and Fig. S1B), and helix α3, belonging to the C-terminal part of the polypeptide, is an important element in defining the intersubunit crevice (see below) (Fig. 5F).

Although in the trimers observed in the different crystals the three subunits were practically identical, some conformational differences were identified among subunits (Fig. 5H). These changes, best represented in the high-resolution structure of SeCutA (crystal SeL3), occur in two parts of the protein chain, in particular residues (SeCutA numbering) 52–57, in the vestigial T-loop, and at residues 81–89, in the B-loop (Fig. 5H), particularly at Pro86 and Tyr87 (respective placements of their Cα atoms of up to 6.1 Å and 4.8 Å, Fig. 5J). When residues 52–57 and 81–89 were excluded from the superimpositions, rmsd values for the three subunits of the trimer found in SeL3 were ≤ 0.54 Å (93 Cα atoms superimposed), clearly illustrating the high structural stability of each subunit.

**Intersubunit cargo-carrying pockets**

CutA proteins exhibit three pockets at intersubunit junctions, where the canonical PII proteins have their nucleotide-binding pockets [3,6,8,17]. Each pocket, reflecting the crevice between adjacent molecular β sheets, is delimited (Fig. 5E,F and Fig. 6A) by the arch β2β3 hairpin of a subunit and the C-terminal helix (helix α3) of the next subunit. In addition, the vestigial T-loop (β2-β3 connector), B-loop (α2-β4 connection), and the β1-α1 connector of the first of these subunits also contribute to delineate the pocket. The NsCutA and SeCutA pockets are negatively charged (Fig. 6B,C) and highly polar, including highly conserved residues Tyr51, Glu60, Glu62, Tyr87, Glu91, and Tyr104 (SeCutA numbering used from here on; see Fig. 5A for correspondences with NsCutA residues) (Fig. 6D,E). These residues largely define an opening (diameter of about 6 Å) deep in the pocket that connects it with the water-filled internal cavity of the trimer (not shown). Cys41 and Lys68 (the only positive residue in the pocket) are also constant and sit near this narrow opening (Fig. 6D). The conservation of these and other residues that form the pocket (Fig. 6E, Fig. S2) suggests important roles for these residues and for the pockets. The negative potential, the pocket size, and the lack of conservation of most ATP/ADP binding residues of canonical PII proteins (Fig. 5A) [28] seem to exclude the binding of these PII effectors to the CutA pockets. Indeed, isothermal titration calorimetry (ITC) failed to reveal any binding of ATP and ADP to NsCutA (data not shown), which are effectors of canonical PII proteins [2,28], and of cAMP, which is the effector of the noncanonical PII-like protein SbtB [7]. Interestingly, in line with the results of our functional in vivo studies, we could not detect also the binding of Cu²⁺ to recombinant CutA protein using ITC, among other tested metal cations. In addition, we could not detect any electron density for ATP or for Mg²⁺ within the SeL0 crystal (1.75 Å resolution), which grew in presence of ATP and Mg²⁺ (10 mM each; Table 1).

The CutA pockets have been proposed to be functional elements that could bind some unrecognized ligand, playing crucial but as yet non-demonstrated CutA function(s) [17]. In agreement with this proposal, the SeL1, SeL2, and SeL3 crystals were found to host in these pockets, respectively, one, two, and three molecules of the Bis-Tris buffer present in the crystallization solutions (shown for SeL3 in Fig. 5E,F and Fig. 6C). Similarly, four of the six subunits in the ASU of the Ns2 crystal hosted a molecule of the MES buffer used in the Ns2 crystallization solution (Fig. 6A,B). The high-resolution structures of Bis-Tris bound to the SeCutA pockets showed (Fig. 6F) the buffer molecule lying on Trp107*, Tyr51, and Ala 40* (the asterisk marks elements from a second subunit) and hydrogen bonding, via its OH groups, to Leu 89, the side-chain carboxylate of Glu62, and, indirectly via a water molecule, to Cys41. Bis-Tris was positioned identically in two pockets of SeL3, while in the third pocket it was in an inverted orientation so that its ternary N and quaternary C atoms revert their respective positions relative to the other orientation. This allows preservation of most contacts, although being mediated by different OH groups of Bis-Tris, and adapts the ligand binding to movement of the B-loop (not shown). MES buffer binds to the pockets in Ns2 so that its morpholino part grossly occupies the position of Bis-Tris in SeCutA, while the negatively charged sulfate stems out from the site (Fig. 6A, B and G), as previously observed in the structure of CutA from *Pyrococcus horikoshii* (PDB: 4NYO) [29]. The same can be said for HEPES buffer, observed binding in the deposited structure (PDB: 3GSD; no publication associated) of CutA from *Yersinia pestis*, where the sulfonic acid also stems out and the remainder of the molecule replaces the Bis-Tris of SeCutA.

In the cyanobacterial structures, Bis-Tris or MES binding did not trigger any chain movements, as shown by superimposition of a chain with Bis-Tris of the SeL3 crystal and another one without Bis-Tris from SeL0. In the latter, structural differences occurred in the same regions and with similar...
Discussion

PII-like proteins are structurally similar and clearly related to canonical PII proteins, but they lack the PII PROSITE signature sequences, and their functions, when known, differ from those of classical PII proteins [6,7,30,31]. Bioinformatics and structural genomics approaches showed that PII-like proteins represent an even more widespread family of trimeric regulators than canonical PII proteins, distributed in almost all living organisms [6,30]. Among noncanonical members of the superfamily, a unique class of PII-like proteins is involved in controlling carbon metabolism, as exemplified by the carboxysome-related PII protein (CPII), which binds ADP/AMP and bicarbonate and was proposed to sense bicarbonate availability [31]. Some of us also characterized recently in cyanobacteria a new PII-like protein named SbtB [7], which senses the second messenger cAMP (in addition to ATP, ADP, and AMP) and which, being involved in the control of the bicarbonate transporter SbtA, links cAMP sensing to CO₂ metabolism [7,32]. Furthermore, in Firmicutes such as *Staphylococcus aureus* or *Bacillus subtilis*, a PII-like protein (termed PstA or DarA) was identified to sense the second messenger c-di-AMP, although its function is still unknown [33–35]. In all these cases, the PII/PII-like proteins exert their regulatory function through binding small effector molecules, mainly adenylate nucleotides, which induces conformational changes to transduce the signal to the PII/PII-like targets [32]. We now deal with another noncanonical PII protein of unclear function, CutA, a protein that is distant in sequence from canonical PII proteins [5] but whose core structure is almost identical to the other PII superfamily members. However, it differs in the effector-binding site and the output domain, the T-loop, a minimal rudiment of the T-loop (termed vestigial T-loop) in CutA (Fig. S1B). These features set CutA apart from the other so-far characterized members of the PII superfamily. However, we could identify the binding pockets of ScCutA and NcCutA, which hosted, respectively, Bis-Tris and MES molecules from the crystallization buffer, at the intersubunit clefts of the trimer. The fact that these pockets are lined by highly conserved residues among CutA proteins that differ from those residues used for the binding of nucleotides in characterized members of the PII protein superfamily signals the importance of these pockets. The respective binding of Bis-Tris and MES molecules to ScCutA and NcCutA implies a tendency of CutA proteins to harbor small organic molecules with polar groups in these crevices, also observed in previous CutA structures from *Pyrococcus horikoshii* (PDB: 4NYO) and *Yersinia pestis* (PDB: 3GSD) bound to MES and HEPES, respectively. However, the natural CutA ligands of these sites as well as the conformational changes potentially triggered remain to be identified.

To our surprise, we were neither able to reveal binding of Cu²⁺ to cyanobacterial CutA proteins *in vitro* by X-ray crystallography nor by ITC and could not show involvement of CutA in heavy metal tolerance *in vivo* by physiological means in three phylogenetically distant bacteria. Actually, Cu²⁺ was previously observed [36] only in the structure of one CutA protein (PDB file 1UKU; for *P. horikoshii* CutA, PDB CutA) among the 18 crystal structures of CutA proteins reported or deposited in the Protein Data Bank (PDB).
(Table S1) belonging to 14 different organisms (two archaea, eight bacteria and four eukaryota, two of them mammals). Even for PhCutA, later crystallization work [29] failed to obtain Cu$^{2+}$-containing structures although good crystals were obtained in a Cu$^{2+}$-containing crystallization solution (PDB file 4NYO).

In addition, metal analysis of EcCutA confirmed previously that the protein in its apo-state was free of any divalent ions (Cu$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Mn$^{2+}$, and Cd$^{2+}$) [8].

Our results in both cyanobacteria and E. coli ruled out a role for the corresponding PII-like CutA proteins in heavy metal resistance, while only the gene product of cutA2 was necessary to confer such heavy metal tolerance to E. coli cells. In addition, we could not find within the S. elongatus genome an orthologous gene for cutA2 (BLASTp search using E. coli CutA2 protein sequence), thus excluding any cutA2 influence. It is worth noting that the previous report for involvement of PII-like EcCutA in heavy metal tolerance [9] was based on the genetic location of the E. coli cutA1 gene (PII-like) next to the suppressor copper sensitivity cutA2 gene, also known as scsB and dsbD [37–39], but that a rigorous physiological examination of the cutA1 mutant was lacking. Therefore, the annotation of the PII-like CutA as divalent ion tolerance protein is both misleading and clearly at odds with our results and those previously obtained in other diverse biological systems. In agreement with our phenotypic observations, the level of gene expression of cutA1 in the plant pathogen Xylella fastidiosa showed no significant change in two different media under elevated levels of external Cu$^{2+}$ concentrations [40], while in the higher plant Arabidopsis thaliana, the cutA knockout lines revealed that AtCutA is not involved in Cu$^{2+}$ tolerance [41].

While our phenotypic analysis of cyanobacterial cutA mutants rule out the involvement of CutA in heavy metal (divalent metal) tolerance, incomplete segregation of the inactivated cutA allele in Nostoc highlights its biological importance for multicellular lifestyle in this cyanobacterium, similar to glmB [42,43] and shtB (ORF all2133; unpublished data from K.A. Selim) encoding for canonical PII and PII-like proteins, respectively, in Nostoc spp. [42]. Altogether, our biochemical and physiological results emphasize the need to search for the signals and effector molecules regulating CutA activity and the specific cellular processes targeted by this enigmatic protein.

In summary, our present and previous structural studies of various proteins of the PII superfamily [2,3,6,7,31–33,44,45], including canonical PII, SbtB, CPII, PstA, and CutA proteins, indicate that the trimeric assembly is a key evolutionary feature of this superfamily. In fact, this trimeric assembly is also found in cases where the PII domain is a regulatory element controlling the oligomeric state of larger, multidomain proteins such as Nif-3 and HisG [46–48]. These features make the members of the PII superfamily a treasure chest for use in protein design and for the development of in vivo and in vitro biosensors. While canonical PII proteins have indeed been used to develop in vivo 2-OG biosensors [49–51], a CutA protein was engineered to produce a novel tetrahedral two-protein co-assembling nanomaterial, with potential use in targeted drug delivery [52,53]. Further bio-physicochemical studies will be necessary for understanding the full breadth of functional roles covered by the PII superfamily in central metabolism and its potential for biotechnological applications.

Materials and Methods

Bioinformatic analysis

The analysis was carried out using tools offered within the MPI Bioinformatics Toolkit [19]. The HHpred search to identify distant homologs of CutA was seeded with the E. coli CutA protein and was performed against the PDB_mmCIF70 (sequences of protein databank structures clustered at 70% sequence identity) profile HMM database using default settings. To gather CutA homologs, we searched the nr90 sequence database, a version of the nonredundant protein sequence database filtered to a maximum pairwise sequence identity of 90%, using two iterations of PSI-BLAST [18], with an E-value inclusion cutoff of 1e-3. The obtained full-length sequences were clustered in CLANS [21] at a P-value cutoff of 1e-32, with attract value = 2 and repulse value = 20.

Generation of cutA knockout and knockdown strains of cyanobacteria

To inactivate cutA in S. elongatus PCC 7942, the wild-type strain was transformed with cosmid 8534-E4 taken from the Unigene set (an existing insertion mutant library for S. elongatus [23]) which carries the cutA ORF inactivated with a Tn5 transposon conferring resistance to kanamycin. Transformation of S. elongatus was achieved using standard protocols [54] and resulted in two classes of transformants: double recombinants that substitute the wild-type allele by the mutant allele, and single recombinants that result from the integration of the entire cosmid with duplication of the targeted gene. The two types of transformants were distinguished by the Cm marker on the cosmid backbone, which is present only in single recombinants. Two clones Kn$^R$ Cm$^5$ were isolated, and the correct inactivation of cutA was verified by PCR with primers 1F and 1R (Table S2). A second similar alternative for knocking out
the *S. elongatus* cutA gene also was successful. In this alternative, the insertion of the kanamycin resistance cassette relied on PCR amplifications using three primer pairs (oligonucleotides used in the present study are shown in Table S2), 1238/1239, 1240/1241, and 1242/1243, on cloning into the pUC19 plasmid using Gibson assembly, and on transformation of *S. elongatus* PCC 7942 with the resulting plasmid, essentially as described for *Synechocystis* sp. PCC 6803 [7]. The mutants were selected on BG11 plates supplemented with 50 μg mL⁻¹ kanamycin and verified by PCR with primer pair 1240/1243.

Attempts to knockout the *alr7093* gene (encoding CutA) of *Nostoc* sp. PCC 7120 used the double recombination strategy and kanamycin selection for recombinants. The pRL277 plasmid hosting the upstream and downstream regions of *alr7093* flanking the kanamycin resistance cassette was introduced into the cyanobacteria via conjugation [26]. Nevertheless, full segregation of *alr7093* mutant alleles was not achieved (Fig. 3A).

For cutA knockdown in *Nostoc* sp. PCC 7120, a previously reported strategy [25,26] was used in which the cells are transformed with *AS cutA*-pAM1956 plasmid (generated based on pAM1956 plasmid [25] hosting in reverse-complement orientation *alr7093* (encoding NtCutA) amplified with the 1178/1179 primer pair, placing the reverted gene under the control of a strong copper-inducible promoter (Pₚₚₑₚₑ), which was cloned using primer pair Fw/1777. The amplified PCR products (Pₚₚₑₚₑ and the reverse-complement *alr7093*) were cloned into pAM1956 using Gibson assembly. In the pAM1956-*cutA* construct, a promoter-less GFPmut2 gene is immediately downstream the antisense producing *cutA* gene and is co-expressed with antisense *cutA* under Pₚₚₑₚₑ control, so that the expression of the downstream green fluorescent protein (GFP) was used as a reporter for production of the *alr7093* antisense-RNA. The *AS cutA* mutant was selected on BG11 plate (without Cu²⁺) supplemented with 50 μg mL⁻¹ kanamycin and verified via PCR with primer pair Fw/1179.

**Assessment of metal tolerance of cyanobacterial cells**

*S. elongatus* strains were routinely grown photoautotrophically on BG11 medium while shaking at 30°C under continuous illumination conditions (~30 μE m⁻² s⁻¹) [55], checking cell density as OD₅₇₀. Culture biomass was estimated as chlorophyll content from the OD₆₆₅ of a centrifuged methanolic extract of 0.1 mL of culture [56]. In one approach to assess metal tolerance, cultures were adjusted to 10 μg chlorophyll mL⁻¹, and then, serial dilutions of the cell suspension were prepared and equal size drops of these dilutions were seeded on solid 1% agar plates of BG11 medium supplemented with CuSO₄, ZnSO₄ or CoCl₂ as indicated. Plates were photographed after 5 days of growth at 30°C under constant illumination. In a second approach, exponentially growing wild-type cells of either *S. elongatus* or *Nostoc* sp. and the respective knockout or knockdown strains were subjected to heavy metals stress by supplementing BG11 media with one of the following heavy metals: Cu²⁺, Fe²⁺, Pb²⁺, Co²⁺, Zn²⁺, Cr³⁺, Cd²⁺, Ni²⁺, and Mn²⁺ (used as the chlorides, except Cu²⁺ and Fe²⁺, which were used as the sulfates) at 2.5–50 μM concentrations in 24-well plates. The survival of heavy metal-stressed cells was checked by the drop-plating assay [7], where drops from each treatment were spotted on BG11 agar plates in absence of heavy metals and incubated at 28°C under a light intensity of 30–50 μmol photons m⁻² s⁻¹ for one week. Growth rates of cells cultivated in liquid medium under stress of 25 μM Cu²⁺ metal were recorded by measuring the increase in optical density at 750 nm (OD₇₅₀) at various time points.

**Escherichia coli copper tolerance assays and complementation studies**

Wild-type *E. coli* (strain BW25113) and the corresponding cutA-lacking strain JW4097 of the Keio collection [27], in which cutA1 is replaced by a kanamycin resistance cassette, were provided by Dr. F.J. Mojica, Universidad de Alicante. Both strains were lysogenized for phage λDE3 using the λDE3 Lysogenization Kit (as recommended in the kit, from Novagen, Merck Life Sciences, Madrid, Spain), thus to allow T7 promoter-dependent expression of pET15b-carried genes. *E. coli* cutA1 and cutA2 genes (EGI1277 and EG12178 EcoGene identification codes, http://ecogene.org/), encoding, respectively, PII-like CutA1 and DsbD, were separately PCR-cloned from genomic DNA of *E. coli* K12 into the NdeI-BamHI sites of pET15b, using in the PCR amplification step Deep Vent DNA polymerase (New England Biolabs) and the primer pair cutA1-NdeI-F/cutA1-BamHI-R for cutA1, and cutA2-NdeI-F/cutA2-BamHI-R for cutA2 (Table S2). The resulting plasmids pETcutA1 and pETcutA2 encode the corresponding proteins N-terminally fused to the MGSSHHHHHHHSGPVPRGS tag. Automated fluorescent Sanger sequencing was used to confirm the correctness of the constructions, the absence of mutations, to determine the insertion point of the kanamycin resistance cassette in the genome of the JW4097 *E. coli* strain, and the lack of insertion of this cassette in the wild-type strain (primers P1 and P2, Table S2, were used for PCR amplification of the region including the insertion point). Standard procedures were used for making electrocompetent cells and for electrottransformation with plasmids, selecting transformants by culturing under aeration at 37°C in LB-ampicillin.

For Cu²⁺ resistance assays, the λDE3-lysogenized BW25113 (WT) and JW4097 cells were grown to identical cell densities (estimated from OD₆₀₀, assuming that 1 OD₅₇₀ corresponds to 10⁶ cells mL⁻¹) [56], and then, they were diluted to the indicated concentrations in sterile water. Equal size drops of these dilutions were seeded on LB-agar supplemented with or without 3 mm
CuSO₄. Growth in individual drops was checked by naked eye after 18 h of plate incubation at 37 °C. Similar assays were carried out with the lysogenized JW4097 strain that had been transformed with either ‘empty’ parental pET15b or the pETcutA1 or pETcutA2 plasmids.

Production of recombinant CutA proteins

For production of SeCutA His₆-tagged N-terminally, the S. elongatus cutA gene (Cytochrome oxidase, Synec-7942_2261), PCR-amplified from genomic DNA using Deep Vent DNA polymerase (New England Biolabs) and the CutA-NdeI-F and CutA-BamHI-R oligonucleotides (Table S2), was cloned into pJET1.2/blunt with the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). Then, after release from this plasmid by NdeI/BamHI double digestion, it was subcloned directionally into the corresponding sites of pET28a (Novagen) using Quick Ligase (New England Biolabs). The resulting plasmid encoding SeCutA N-terminally fused to the MGSSHHHHHHSSGLVPRGSH tag was used to transform E. coli BL21(DE3) cells (Invitrogen), which were grown at 37°C to a cell density of 0.7 OD₅₅₀ in 0.5-L well-aerated cultures in liquid LB-kanamycin (50 µg·mL⁻¹) medium. Then, the temperature was lowered to 20°C, 0.1 mM IPTG was added, and the culture was continued at 20°C for 20 h.

Subsequent steps were at 0°C. Centrifugally harvested cells from a 500-mL culture were suspended in 12 mL of 0.1 M Tris/HCl pH 8.0, 0.15 M NaCl, and 1 mM EDTA and were disrupted by sonication and centrifuged (30 min, 13 500 g), and the supernatant was applied to a 1-mL Ni-chelate HisTrap-ruptured by sonication and centrifuged (30 min, 13 500 g), and the supernatant was applied to a 1-mL Ni-chelate HisTrap (Thermo Fisher Scientific). Then, after release from this plasmid by NdeI/BamHI double digestion, it was subcloned directionally into the corresponding sites of pET28a (Novagen) using Quick Ligase (New England Biolabs). The resulting plasmid encoding SeCutA N-terminally fused to the MGSSHHHHHHSSGLVPRGSH tag was used to transform E. coli BL21(DE3) cells (Invitrogen), which were grown at 37°C to a cell density of 0.7 OD₅₅₀ in 0.5-L well-aerated cultures in liquid LB-kanamycin (50 µg·mL⁻¹) medium. Then, the temperature was lowered to 20°C, 0.1 mM IPTG was added, and the culture was continued at 20°C for 20 h.

Subsequent steps were at 0°C. Centrifugally harvested cells from a 500-mL culture were suspended in 12 mL of 0.1 M Tris/HCl pH 8.0, 0.15 M NaCl, and 1 mM EDTA and were disrupted by sonication and centrifuged (30 min, 13 500 g), and the supernatant was applied to a 1-mL Ni-chelate HisTrap-HP column fitted in an AKTA-FPLC system (both from GE Healthcare), followed by a 30-mL wash with the same buffer with the EDTA replaced by 20 mM imidazole. SeCutA was eluted with an 80-mL linear gradient of this buffer containing 20–500 mM imidazole, collecting fractions. The purest fractions (purity monitored by SDS/PAGE) were pooled, the imidazole was removed, and the protein was concentrated to ≥ 7 mg protein·mL⁻¹ by centrifugal ultrafiltration (Amicon Ultra, from Millipore; 10 kDa cutoff membrane).

For production of C-terminally Strep-tagged CutA proteins from Nostoc sp. PCC 7120 (NstCutA; encoded by ORF alr7093) and from S. elongatus, the corresponding genes were cloned into pASK-IBA3 as described previously [7,57,58], using, respectively, primer pairs 1414/1415 and 1314/1315 (Table S2). The expression and purification of these strep-tagged CutA proteins were also achieved as described [7,57-59].

Protein crystallization, X-ray diffraction, and structure determination

Crystallization trials were performed via vapor diffusion at 20–21°C in 96-well sitting-drop plates hosting drops composed of 0.4 µL of reservoir solution (Table 1) and 0.4 µL of protein solution containing either 7–10 mg·mL⁻¹ of SeCutA (His-tagged) in 0.1 M Tris/HCl pH 8.0, 0.15 M NaCl, or 20 mg·mL⁻¹ of NstCutA in 25 mM Tris/HCl pH 8.0, 37.5 mM NaCl. SeCutA yielded crystals (called here SeL0, SeL1, SeL2, and SeL3, to reflect the number of Bis-Tris molecules bound per trimer, see below) under four conditions (Table 1). For NstCutA, orthorhombic (Ns1) and trigonal (Ns2) crystal forms were obtained with the reservoir solutions listed in Table 1. Diffraction experiments were conducted at 100 K using the synchrotron beamlines and detectors indicated in Table 1.

For SeCutA, data were obtained at up to 1.17–2.00 Å resolution and were processed and scaled with XIA2 [60] and AIMLESS [61] (Table 1). Phasing was achieved by molecular replacement with Phaser [62,63] using a single subunit of E. coli CutA (PDB 1NAQ) [8] as search model, yielding one CutA homotrimer in the ASU. After refinement (see below), single-subunit models from these crystals were used for phasing in the other crystals of the same protein, yielding single trimers in the ASUs of the other three crystals of SeCutA. Rigid body refinement with REFMAC5 [64] (used for optimizing the positions of the three subunits in each trimer and also for model refinement) was followed by alternative iteratively cycles of restrained refinement and of manual model building with Coot [65], with incorporation of ligands when necessary. Isotropic B factors and anisotropic refinement (with REFMAC5) were used in the last steps of refinement of SeL2, whereas for SeL0, SeL1, and SeL3, TLS was used in the last refinement steps, the TLS groups being chosen with the TLSMD server (http://skuld.bmsc.washington.edu/~tlsmd/). The stereochemistry of each model was checked and improved with PDBePDB_REDO [66].

Data obtained for the two NstCutA crystal forms were integrated and scaled using XDS [67] (Table 1). Molecular replacement with MOLREP [68] using monomers of Thermus thermophilus CutA (PDB: 1V6H) as a search model located six chains in the ASU of both crystal forms. After initial rigid body refinement with REFMAC5 [64], chain F of crystal Ns2 was poorly defined in the electron density but could be refined using NCS constraints; both structures were completed via cyclic manual modeling with Coot and refinement with REFMAC5.

For all datasets, all diffraction data were used throughout the refinement processes, except the 5% randomly selected data used to calculate the Rₓᵧᵧ. Geometry analysis of the protein main chain torsion angles with Rampage [69] and PROCHECK [70] revealed excellent values for the models. Refinement and Ramachandran statistics are shown in Table 1.

Other methods

Size-exclusion chromatography of His-tagged SeCutA (0.02–0.1 mg applied in 0.05 mL) or of standards of known mass was carried out on a Superdex™ 75 10/300 GL column mounted on a ÄKTA FPLC system (column and
system from GE Healthcare; Barcelona, Spain) and run with a solution of 0.1 M Tris/HCl pH 8/0.15 M NaCl delivered at a flow rate of 0.4 mL·min⁻¹, monitoring continuously the optical absorption of the effluent at 280 nm. A semilogarithmic plot of molecular mass (logarithmic scale) versus elution volume (linear scale) was used to estimate the oligomeric nature of SeCutA in solution. Alternatively, when indicated, a Superdex™ 200 PC 3.2/30 column (2.4 mL) was used as previously reported [7,57,58].

Isothermal titration calorimetry was performed using VP-ITC (MicroCal, Malvern Panalytical, Malvern, UK) as described previously [7,57,59], after exhaustive dialysis of CutA protein versus 25 mM Tris/HCl pH 7.8 supplemented with 200 mM NaCl and Chelex 100 to remove any contamination of divalent cations.

Protein was assayed according to Bradford [71], with a commercial reagent from Bio-Rad (Alcobendas, Spain), using bovine serum albumin as a standard. SDS/PAGE was carried out in 15% polyacrylamide gels with Coomassie staining [72]. Amino acid sequence alignments were performed with Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Graphical representations of protein structures were prepared with PyMOL (http://www.pymol.org/) or UCSF Chimera (http://www.rbvi.ucsf.edu/chimera). Root mean square deviation (rmsd) values were calculated by superimposition of structures using Coot [65].

Acknowledgments

This work was supported by grants from DFG to K.F. (Fo195/9-2, RTG 1708-2), from DAAD to K.A.S, from the Spanish Government to V.R. and C.M-M. (BFU2014-58229-P, BFU2017-84264-P) and to A.C. (BFU2015-66360-P), and from BioStruct-X (EU) to V.R. for synchrotron access (grant agreement N°283570, proposal 7687), and by Open Access Publishing of Tübingen University. The authors are thankful to Reinhard Albrecht and Nadine Gougeard for technical support, crystallographic sample preparation and assistance with diffraction data collection, and to the IBV-CSIC crystallographic facility for crystal growth. We are grateful to the staff of beamline X10SA/SLS, Diamond (Oxfordshire, UK) and Alba (Barcelona, Spain) synchrotrons for excellent technical support. Open access funding enabled and organized by Projekt DEAL.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

KAS, LT, KF, and VR conceived and initiated the project. VA performed and wrote the bioinformatic analysis. KAS, JE, and AC generated the different cutA knockout mutants of *S. elongatus*, while KAS generated the cutA knockout mutant in *Nostoc*. KAS and LT designed, performed, and interpreted the biochemical, structural, and physiological experiments, with inputs from VR, JE, and AC to LT in the analysis and interpretation of *S. elongatus* experiments performed in Spain. CM-M and MDH supported the structural studies (X-ray crystallography). KAS, MDH, KF, LT, CM-M, and VR analyzed data. KAS, LT, and VR wrote advanced drafts of the manuscript. VR merged the final version of the manuscript, while CM-M and KAS were highly involved with making and editing the figures and tables. All authors but particularly MDH and KF commented and edited on the manuscript, analyzed the results, and approved the final version of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Previously determined CutA structures.

Table S2. Oligonucleotides used in the present study.

Fig. S1. Structural superpositions of NsCutA with EcCutA, with canonical PII and with the SbtB non-canonical member of the PII superfamily.

Fig. S1. Alignment of amino acid sequences of CutA proteins from different organisms.