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The domain of unknown function DUF1521 exhibits metal ion-inducible autocleavage activity – a novel example from a putative effector protein of *Vibrio coralliilyticus* ATCC BAA-450

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Abstract

Vibrio coralliilyticus ATCC BAA-450 is a pathogen causing coral bleaching at elevated seawater temperatures. Based on the available genome sequence, the strain has a type III secretion system. Within the corresponding gene cluster, VIC_001052 is encoded, which contains a conserved domain of unknown function DUF1521. In this study, we show that the purified domain exhibits autocleavage activity in the presence of several divalent metal ions, for example, calcium and manganese but not with magnesium or zinc. Autocleavage is not affected by temperatures between 0 and 30 °C, indicating that seawater temperature is not a critical factor for this activity. The DUF1521 domain and the cleavage site are conserved in several proteins from proteobacteria, suggesting a similar cleavage activity for these proteins.

Introduction

Vibrio coralliilyticus strains are widespread pathogens, which were first isolated from diseased corals in the Indo-Pacific (Ben-Haim *et al.*, 2003a; Sussman *et al.*, 2008). They are considered as the causative agent of coral bleaching and coral white syndrome but were also isolated from other organisms (Ben-Haim *et al.*, 2003a, b; Sussman *et al.*, 2008). Coral bleaching results from the loss or degradation of the photosynthetic active microalgal endosymbionts (Hayes & Bush, 1990; Rosenberg *et al.*, 2009). *Vibrio coralliilyticus* ATCC BAA-450 (*Vc*450) shows a temperature-dependent virulence on *Pocillopora damicornis* (Ben-Haim *et al.*, 2003b). Under laboratory conditions, infection with *Vc*450 leads to coral lysis only at water temperatures of $\geq$ 27 °C (Ben-Haim *et al.*, 2003b). Proteome analysis of *Vc*450 revealed the temperature-dependent induction of a high number of genes involved in virulence (Kimes *et al.*, 2012). Based on genome data (Kimes *et al.*, 2012), *Vc*450 also encodes a type III secretion system (T3SS); however, its contribution to virulence is not known yet. T3SSs are widespread in Gram-negative pathogenic bacteria and *Rhizobiaceae* and translocate proteins from the bacterial cytosol directly into the eukaryotic host cell (Deakin & Broughton, 2009; Büttner, 2012). These so-called effector proteins then interfere with eukaryotic signaling pathways and defense response. The gene cluster encoding a T3SS in *Vc*450 (locus tags *VIC_001039-001056*) is located on a megaplasmid (Kimes *et al.*, 2012). One protein encoded inside this cluster (locus tag *VIC_001052*) shows similarity to the NopE1 and NopE2 proteins from *Bradyrhizobium japonicum* USDA110 (Schirrmeister *et al.*, 2011), which is a symbiont of soybean and a few other legumes. The NopE proteins are type III-secreted proteins and depending on the host plant have a positive or negative effect on symbiosis (Wenzel *et al.*, 2010). Both proteins contain two domains of unknown function (DUF1521), which exhibit a calcium-dependent autocleavage activity (Wenzel *et al.*, 2010; Schirrmeister *et al.*, 2011). In this study, the autocleavage activity of the DUF1521 domain of VIC_001052 (ZP_05884569.1) was analyzed.

Materials and methods

Construction of expression plasmids

Plasmids used in this study were propagated in *Escherichia coli* DH10B (Life Technologies). The *VIC_001052* gene

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was synthesized on the basis of the *E. coli* codon usage (Life Technologies). DNA fragments for the expression of VIC_001052 variants were amplified by polymerase chain reaction using *Pfu* polymerase (Thermo Scientific). The forward primer VicDUFM_for (*aaaa*catatgagctatgaaccgag cagcgg) contains a NdeI site (underlined), the reverse primers VicDUFM_rev (*aaa*gcggccgcttaggtttcaatatcattcacaa tggcatc) and VicDUFMalE_rev (*atat*gcggccgcttagttattatatt ctttcacttccagatcacc) contain NotI sites (underlined), which were used for cloning into the vector pMal-c5x (New England Biolabs). The vector provides cytoplasmic localized MalE protein as N-terminal fusion partner. To support efficient restriction of the amplified fragments, nucleotides were added at the 5′-end (in italics). A combination of the primers VicDUFM_for and VicDUFMalE_rev yielded a fragment coding for a truncated DUF1521 domain of VIC_001052 (amino acid position 73–192 with respect to the full-length protein). Cloning resulted in plasmid pVCD011 encoding protein MVic73–192. A combination of the primers VicDUFM_for and VicDUFM_rev yielded a fragment coding for the complete DUF1521 domain of VIC_001052 (amino acid position 73–243 with respect to the full-length protein). Cloning resulted in plasmid pVCD012 (encoding protein MVic73–243). Plasmid pVCD013 differs from plasmid pVCD012 by encoding a DUF1521 domain (protein MVic73–243D116A), with a replacement of an aspartic acid residue by alanine at position 116 (with respect to the full-length protein).

Protein expression and purification

The DUF1521 domain variants were expressed in *E. coli* BL21 (DE3) (Novagen) as fusions with the maltose-binding protein. Strains were grown in lysogeny broth (LB) medium containing ampicillin (100 μg μL^{-1}). After induction with IPTG (100 μM), cultures were grown at 20 °C for 5 h. Cell crude extracts were obtained as described by Schirrmeister *et al.* (2011) using TKE buffer (50 mM Tris, 200 mM KCl, 10 mM EDTA, pH 8.0). The fusion proteins were purified by affinity chromatography with MBPTrap HP columns (GE healthcare) using TKE buffer (pH 8.0) for loading and 10 mM maltose in TKE for elution.

Protein characterization

Autocleavage was analyzed by incubating the protein (*c.* 5 μg) in TKE buffer supplemented with $CaCl_2$ (25 mM final concentration) for 30 min at room temperature. The reaction was stopped by addition of EDTA to a final concentration of 100 mM.

For determination of the metal ion specificity, the fusion protein was dialyzed against MOPS-sodium acetate buffer (pH 7) and subsequently incubated for 30 min in the presence of the metal ion (2.5 mM) to be tested.

Tests concerning the thermostability of the cleavage reaction were carried out at pH 8.0 in adjusted Tris buffers. The protein solution was pre-incubated for 10 min at the selected temperature, then pretempered $CaCl_2$ solution was added, and the protein was incubated for 30 min. The reaction was stopped by addition of EDTA (100 mM final concentration). Protein samples were analyzed by SDS-PAGE (Lämmli, 1970). For each sample, *c.* 5 μg protein was loaded on a 15 % polyacrylamide gel. PageRuler™ Unstained Protein Ladder (Thermo Scientific) was loaded for molecular weight estimation. Gels were stained after electrophoresis with Coomassie brilliant blue R250.

N-terminal sequencing by Edman degradation

Purified MVic73–243 was incubated with 25 mM $CaCl_2$ for 30 min at 23 °C and afterwards loaded on a NuPAGE™ Novex 8% BisTris Midi Gel (Life Technologies). After electrophoresis, proteins were blotted onto a polyvinylidene diflouride membrane (Hybond P; GE Healthcare) in a semidry blotting chamber (1.5 mA cm^{-2}, 1 h) using NuPAGE™ Transfer Buffer (Life Technologies). The relevant band on the Ponceau stained and dried membrane was analyzed by ChromaTec (Greifswald, Germany).

Bioinformatics and statistical analyses

For retrieval of sequences, blast searches, and domain predictions, the resources of the National Center for Biotechnology Information and the Motif Scan tool of MyHits (Pagni *et al.*, 2007; Johnson *et al.*, 2008) were used. DUF1521 domains were aligned with MultAlin (Corpet, 1988). The cellular localization of the VIC_001052 protein was predicted with PSORTb (Yu *et al.*, 2010).

Results

The DUF1521 domain of VIC_001052 exhibits autocleavage within a conserved GDPH motif

The hypothetical protein VIC_001052 has a size of 370 amino acid residues. Using RPS-Blast (Marchler-Bauer & Bryant, 2004), a partial DUF1521 domain comprising about 120 amino acid residues (position *c.* 73–192) was predicted (Fig. 1a). The application of the Motif Scan tool (see Materials and methods) indicated that the domain might extend up to amino acid position 246. The DUF1521 domain of NopE1 from the symbiont *B. japonicum* exhibits autocleavage in the presence of calcium. To test if this

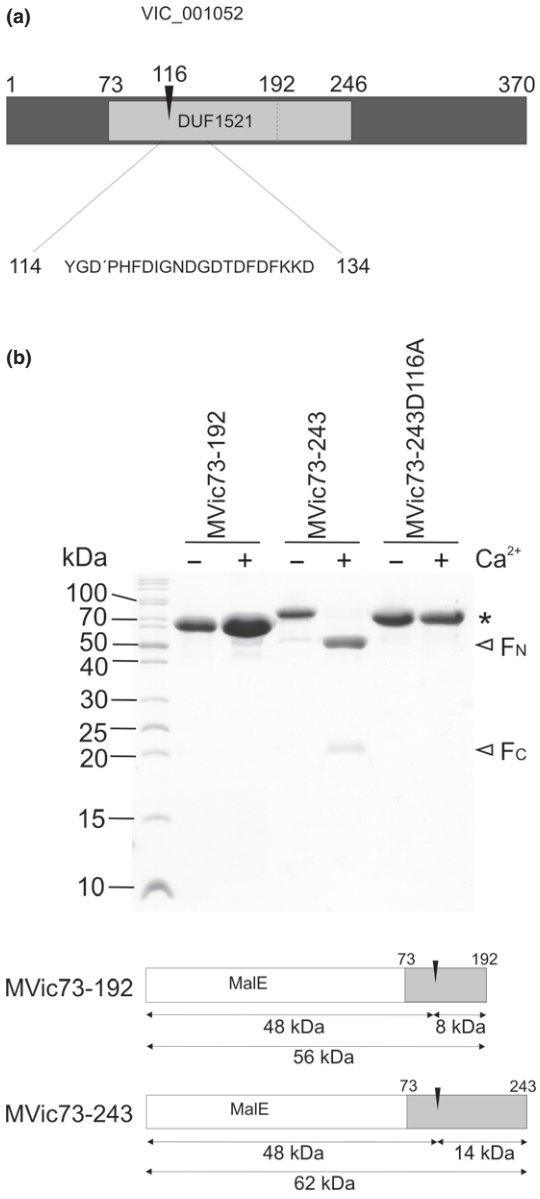

Fig. 1. Autocleavage activity of DUF1521 fragments of the VIC_001052 protein. (a) Scheme depicting the VIC_001052 protein (dark gray box) and the position of the DUF1521 domain (light gray box). Numbers refer to amino acid positions within the protein. The predicted cleavage site and the neighboring aspartate-rich region are depicted below. The filled arrowhead indicates the cleavage site (aa 116). (b) Autocleavage of different DUF1521 fragments incubated without (−) or with (+) $CaCl_2$. Proteins analyzed are depicted in the lower part. Numbers refer to amino acid positions within VIC_001052. The gray shading indicates the DUF1521 region. Calculated molecular weights of the full-length proteins and fragments are given. MVic73–243D116A is identical to MVic73–243 with the exception that it contains an alanine instead of an aspartate residue at position 116. Protein fragmentation was analyzed by SDS-PAGE. The full-length protein is marked with an asterisk (*). Open arrowheads indicate the position of the resulting cleavage products. F_N, N-terminal fragment; F_C, C-terminal fragment.

activity is preserved in the DUF1521 domain of VIC_001052, two different fragments were expressed in *E. coli* as fusions with the MalE protein (MVic73–192 and MVic73–243). Because first experiments indicated that the MalE protein does not influence cleavage activity of the DUF1521 domain (data not shown), all data presented here were generated with the fusion proteins. The purified protein MVic73–192 did not show any fragmentation in the presence of calcium (Fig. 1b). However, MVic73–243 was cleaved into two fragments after addition of calcium, suggesting that the larger fragment contains a functional domain. The fusion proteins as well as the C-terminal cleavage product (F_C) had a lower electrophoretic mobility during SDS-PAGE than expected (Fig. 1b). The calculated molecular weights for MVic73–192 and MVic73–243 are 48 kDa (observed *c.* 60 kDa) and 62 kDa (observed *c.* 71 kDa), respectively, and 14 kDa for the C-terminal fragment F_C (observed *c.* 22 kDa).

N-terminal sequencing of the fragment F_C revealed the sequence PxFD, indicating that the cleavage site is localized in the unique sequence GD'PHFD. In order to test if the aspartic acid residue is essential for cleavage, it was replaced by alanine, resulting in protein MVic73–243D116A, which was not fragmented (Fig. 1b). This shows that the aspartate is crucial for function. An alignment of all DUF1521 domain sequences available in GenBank revealed that the GDPH motif is conserved (Supporting Information, Fig. S1), suggesting a similar self-cleavage activity for all these proteins.

Effect of temperature and metal ions on autocleavage

To test if the cleavage activity can be induced by metal ions other than calcium, protein MVic73–243 was incubated with $CdCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, $MnSO_4$, $NiCl_2$, or $ZnSO_4$. Previously, the interference of buffer substances in metal-binding assays was reported (Fischer *et al.*, 1979). Especially, Cu(II) and Zn(II) can form complexes with Tris buffer. Therefore, the metal dependancy of autocleavage was analyzed in MOPS buffer. This buffer was recommended for binding studies, as it does not coordinate metal ions significantly (Kandegedara & Rorabacher, 1999). Incubation with manganese (II) or calcium ions led to nearly complete fragmentation of MVic73–243 (Fig. 2). Some uncleaved protein was still visible after incubation with $CdCl_2$ or $CoCl_2$. Significant autocleavage activity was also observed in the presence of $NiCl_2$. Protein samples incubated with $MgCl_2$, $CuCl_2$, or $ZnSO_4$ show faint bands for the N-terminal fragment (F_N). In all protein preparations, we observe some cleavage products without addition of any metal ions, indicating that cleavage either takes place already in the bacterial cell during expression or

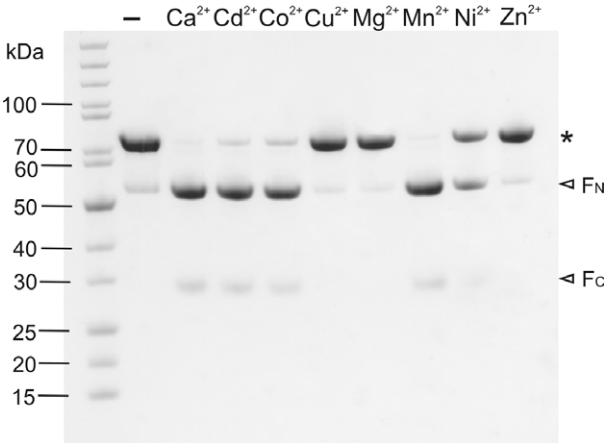

Fig. 2. Metal ion-induced autocleavage of MVic73–243 in MOPS buffer. The purified protein was incubated without (–) or with the indicated metal ions at a final concentration of 2.5 mM. Fragmentation was analyzed by SDS-PAGE (NuPAGE, 8% Bis-Tris gel; Life technologies). The full-length protein is marked with an asterisk (*). Open arrowheads indicate the position of cleavage products. F_N, N-terminal fragment; F_C, C-terminal fragment.

during the isolation procedure. Therefore, we do not think that $MgCl_2$, $CuCl_2$, or $ZnSO_4$ promote cleavage significantly, although we observed weak cleavage with Cu^{2+} in Tris buffer (data not shown).

Vibrio coralliilyticus is a marine pathogen. To determine if temperature affects autocleavage activity of the DUF1521 domain, MVic73–243 was incubated with calcium solution at 0, 10, 20, and 30 °C. Cleavage was similar at all tested temperatures (Fig. 3), suggesting that seawater temperature might not influence the activity of this protein.

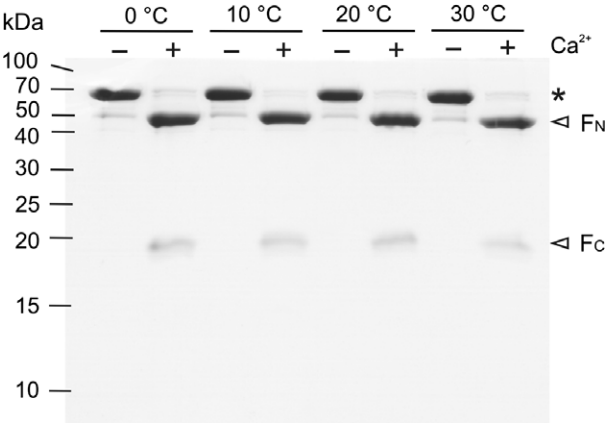

Fig. 3. Autocleavage of MVic73–243 at different temperatures. Purified protein was incubated without (–) or with (+) $CaCl_2$ for 30 min at the indicated temperatures. Fragmentation was analyzed by SDS-PAGE. The full-length protein is marked with an asterisk (*). Open arrowheads indicate the position of cleavage products. F_N, N-terminal fragment; F_C, C-terminal fragment.

Discussion

The full-length proteins studied here and the C-terminal cleavage product migrated significantly slower in an SDS polyacrylamide gel than expected from their predicted molecular weights. Highly charged proteins have been reported to exhibit migration anomalies in SDS gels (Graceffa *et al.*, 1992; Hu & Ghabrial, 1995; Dogra *et al.*, 2012). For example, β-lactamase of *Actinomadura* R39 appears as a *c.* 55 kDa protein after SDS-PAGE but has a calculated molecular weight of 29 kDa (Matagne *et al.*, 1991). This anomalous behavior of the protein is due to its strong negative charge (pI < 4). The most abundant amino acids in the studied DUF1521 domain are aspartate (14%) and glutamate (10.5%), which are probably the cause for the observed mobility anomaly of the *Vc*450 proteins and the F_C fragment (pI values *c.* 4.0–4.5).

Unlike the described spontaneous degradation of aspartate residues in peptide sequences, which is a slow process taking days (Piszkiewicz *et al.*, 1970; Geiger & Clarke, 1987; Oliyai & Borchardt, 1993), the cleavage of the DUF1521 domain of VIC_001052 is a fast and metal ion-dependent reaction. The metal ion-induced cleavage of peptide bonds was observed previously (Humphreys *et al.*, 1999; Andberg *et al.*, 2007; Kopera *et al.*, 2012). The cleavage of peptide bonds by metal ions often follows an oxidative mechanism, requiring metal ions, ascorbic acid, and hydrogen peroxide (Andberg *et al.*, 2007). These conditions are rather harsh and probably do not have physiological relevance. A hydrolysis mechanism for the cleavage of peptide bonds has been described by association with soft or borderline metal ions. Serine/threonine- and histidine-containing peptides are specifically cleaved in the presence of nickel ions (Kopera *et al.*, 2010, 2012; Krezel *et al.*, 2010). In the hexapeptide SRHWAP, the imidazole side chain of histidine is responsible for the coordination of the metal ion. Via formation of an intermediate ester with the serine side chain, the peptide bond is cleaved (Kopera *et al.*, 2012). In contrast, the cleavage of VIC_001052 is not a reaction of a short sequence stretch; it depends on the presence of the entire DUF1521 domain as in protein MVic73–243. A truncated version of the DUF1521 domain comprising 120 amino acids (MVic73–192) was not able to undergo autocleavage at position D116. In similar experiments, the minimal fragment for autocleavage within the DUF1521 domain of NopE1 was determined with *c.* 140 amino acids (Schirrmeister *et al.*, 2011).

A calcium-induced autocleavage activity was observed for FrpC a repeat in toxin protein of *Neisseria meningitidis*.

FrpC is cleaved at an aspartate-proline bond in the motif YDPLA (Osička *et al.*, 2004). A domain of about 243 amino acids, which has no sequence similarity to the DUF1521 domain, is essential for autocleavage (Sadilkova *et al.*, 2008). Several EF-hand like motifs are present in FrpC, which are candidates for calcium binding (Osička *et al.*, 2004). In the DUF1521 domain, we found no canonical EF-hand motif. However, a number of proteins that have no EF-hand motif or no canonical EF-hand motif exhibit calcium binding (McPhalen *et al.*, 1991; Gifford *et al.*, 2007). For example, DxDxDG-like sequences are known as calcium-binding sites in several proteins (Rigden & Galperin, 2004). Thus, the aspartate-rich sequence (DGDTD, position 124–128) close to the cleavage site (D116) of VIC_001052 might be involved in coordination of cations. Interestingly, cleavage is also induced by manganese and less efficiently by cobalt and nickel ions but not by magnesium ions, which is most abundant in the cell. This is different to the DUF1521 domain of NopE1, which is cleaved only in the presence of calcium (Schirrmeister *et al.*, 2011). If the broader ion susceptibility of the DUF1521 domain of VIC_001052 is of biological relevance is unclear. The VIC_001052 protein is encoded in the same cluster as the type III secretion system. Bioinformatics analysis with PSORTb (Yu *et al.*, 2010) predicts an extracellular localization. Therefore, VIC_001052 might be a type III–secreted effector protein as is the case for NopE1 of the symbiont *B. japonicum*.

The DUF1521 domain is present in several proteins from α-, β-, γ-, and δ-proteobacteria (Fig. S1). All domains contain the cleavage site motif and further highly conserved amino acid positions. Based on the data presented here, we suggest renaming the domain of unknown function DUF1521 as metal ion-inducible autocleavage (MIIA) domain.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of DUF1521 domains.