Characterization of the Self-Cleaving Effector Protein NopE1 of *Bradyrhizobium japonicum*\(^\dagger\)

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NopE1 is a type III-secreted protein of the symbiont *Bradyrhizobium japonicum* which is expressed in nodules. *In vitro* it exhibits self-cleavage in a duplicated domain of unknown function (DUF1521) but only in the presence of calcium. Here we show that either domain is self-sufficient for cleavage. An exchange of the aspartic acid residue at the cleavage site with asparagine prevented cleavage; however, cleavage was still observed with glutamic acid at the same position, indicating that a negative charge at the cleavage site is sufficient. Close to each cleavage site, an EF-hand-like motif is present. A replacement of one of the conserved aspartic acid residues with alanine prevented cleavage at the neighboring site. Except for EDTA, none of several protease inhibitors blocked cleavage, suggesting that a known protease-like mechanism is not involved in the reaction. In line with this, the reaction takes place within a broad pH and temperature range. Interestingly, magnesium, manganese, and several other divalent cations did not induce cleavage, indicating a highly specific calcium-binding site. Based on results obtained by blue-native gel electrophoresis, it is likely that the uncleaved protein forms a dimer and that the fragments of the cleaved protein oligomerize. A database search reveals that the DUF1521 domain is present in proteins encoded by *Burkholderia phytofirmans* PsNJ (a plant growth-promoting betaproteobacterium) and *Vibrio coralliilyticus* ATCC BAA4450 (a pathogenic gammaproteobacterium). Obviously, this domain is more widespread in proteobacteria, and it might contribute to the interaction with hosts.

Type III secretion systems (T3SS) are specialized protein export machineries of Gram-negative bacteria which deliver effector proteins directly into host cells (10). They have been identified in pathogens and rhizobia (12). In pathogens, effectors play important roles in virulence (9). In rhizobia, effector proteins directly into host cells (10). They have been identified in pathogens and rhizobia (12). In pathogens, effector proteins play important roles in virulence (9). In rhizobia, effector proteins play important roles in virulence (9). In rhizobia, effector proteins play important roles in virulence (9). In rhizobia, effector proteins play important roles in virulence (9). In rhizobia, effector proteins play important roles in virulence (9). In rhizobia, effector proteins play important roles in virulence (9). In rhizobia, effector proteins play important roles in virulence (9).

Alanine scanning mutagenesis revealed that only the aspartic acid and proline residues adjacent to the cleavage sites at the conserved motif GDP/PHV are essential for cleavage (31). A noncleavable NopE1 derivative seems to have lost its physiological function in symbiosis (31).

The NopE1 protein has no characterized homologues in databases. Here we report the analysis of the self-cleavage activity of NopE1 with respect to its sensitivity to protease inhibitors, high and low temperatures, and pH range. The effects of different divalent cations are evaluated, and the essential domain for autoprocessing is determined by deletion analysis.

**MATERIALS AND METHODS**

**Generation of NopE1 derivatives.** Plasmids used in this study are listed in Table 1 and were propagated in *Escherichia coli* DH10B (Invitrogen). Point mutations were generated according to the QuikChange protocol (Stratagene) using pBJD216 and pBJD248 as a template. For the generation of plasmids encoding N-terminally truncated NopE1 versions, appropriate DNA fragments were amplified by PCR and cloned into the expression vector pGEX-4T-3, thereby generating translational fusions with gst. In order to construct C-terminally truncated protein variants, a linker was inserted into pBJD216 immediately downstream of the 3′ end of the coding sequence of *nopE1*, yielding the plasmid pBJD234. The linker contains the recognition sites for XbaI, SaeI, and stop codons in all three reading frames immediately downstream of the SaeI recognition site. pBJD234 was digested with XbaI, SaeI, and exonuclease III, which partially deleted the *nopE1* coding sequence from the 3′ end. The DNA was treated with S1 nuclease and T4 DNA ligase and subsequently transformed into *E. coli*, yielding the plasmids listed in Table 1 (pBJD239 to pBJD242 and pBJD750 to pBJD754). The *nopE1* part of all constructs was verified by nucleotide sequencing.
### TABLE 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX4-T3</td>
<td>Vector for expression of GST-fusion proteins, Ap r</td>
</tr>
<tr>
<td>pBJD216</td>
<td>Encoding a GST-NopE1 fusion in vector pGEX4-T3, Ap r</td>
</tr>
<tr>
<td>pBJD216D147N</td>
<td>Derivative of pBJD216 with an amino acid exchange in NopE1 (D147N), Ap r</td>
</tr>
<tr>
<td>pBJD216D147E</td>
<td>Derivative of pBJD216 with an exchanged amino acid in NopE1 (D147E), Ap r</td>
</tr>
<tr>
<td>pBJD239</td>
<td>Derivative of pBJD234 encoding a C-terminally truncated NopE1 (GST-NopE1 1–452), Ap r</td>
</tr>
<tr>
<td>pBJD240</td>
<td>Derivative of pBJD234 encoding a C-terminally truncated NopE1 (GST-NopE1 1–387), Ap r</td>
</tr>
<tr>
<td>pBJD241</td>
<td>Derivative of pBJD234 encoding a C-terminally truncated NopE1 (GST-NopE1 1–357), Ap r</td>
</tr>
<tr>
<td>pBJD242</td>
<td>Derivative of pBJD234 encoding a C-terminally truncated NopE1 (GST-NopE1 1–282), Ap r</td>
</tr>
<tr>
<td>pBJD248</td>
<td>Derivative of pBJD216 encoding an N-terminally truncated NopE1 (GST-NopE1 315–484), Ap r</td>
</tr>
<tr>
<td>pBJD248D365A</td>
<td>Derivative of pBJD248 with an amino acid exchange in NopE1 (D365A), Ap r</td>
</tr>
<tr>
<td>pBJD248D367A</td>
<td>Derivative of pBJD248 with an amino acid exchange in NopE1 (D367A), Ap r</td>
</tr>
<tr>
<td>pBJD248D371A</td>
<td>Derivative of pBJD248 with an amino acid exchange in NopE1 (D371A), Ap r</td>
</tr>
<tr>
<td>pBJD750</td>
<td>Derivative of pBJD234 encoding a C-terminally truncated NopE1 (GST-NopE1 1–255), Ap r</td>
</tr>
<tr>
<td>pBJD752</td>
<td>Derivative of pBJD234 encoding a C-terminally truncated NopE1 (GST-NopE1 1–246), Ap r</td>
</tr>
<tr>
<td>pBJD754</td>
<td>Derivative of pBJD234 encoding a C-terminally truncated NopE1 (GST-NopE1 1–240), Ap r</td>
</tr>
<tr>
<td>pBJD900</td>
<td>Derivative of pBJD216 encoding an N-terminally truncated NopE1 (GST-NopE1 324–484), Ap r</td>
</tr>
</tbody>
</table>

Apr, ampicillin resistance.

### Results

Protein expression, purification, and characterization. NopE1 and its variants were expressed as glutathione S-transferase (GST) fusion proteins in *E. coli BL21(DE3) (Novagen)*. Purification by glutathione affinity chromatography was done as described previously (31). NopE1 variants resulting from site-specific mutagenesis or partial sequence deletions were analyzed as GST fusion proteins. Further biochemical characterization (temperature and pH stability, oligomerization status, and metal ion specificity) was performed with purified NopE1 after removing the fusion partner GST by thrombin cleavage “on column.” If not otherwise stated, self-cleavage was tested by mixing CaCl$_2$ (final concentration, 25 mM) with 5.5 μg of protein in TKE buffer (50 mM Tris, 200 mM KCl, 10 mM EDTA, pH 7.5) and incubation for 30 min at room temperature. The reaction was stopped by addition of EDTA to a final concentration of 25 mM. For the determination of metal ion specificity, NopE1 was incubated for 30 min in 50 mM Tris buffer (pH 7.5) in the presence of the metal ion to be tested (25 mM, final concentration).

The thermostability of NopE1 was analyzed at pH 6.8. To obtain the correct pH at the indicated temperature, Tris buffers were adjusted between pHs 7.5 and 9.0 (at 20°C). The pH-adjusted protein solution was preincubated for 5 min at the indicated temperature. Then, preheated calcium solution was added and the protein was incubated at the indicated temperature for 30 min. In order to test the autocleavage reaction at different pH values, purified NopE1 was diluted in the following buffers: acetic acid-acetate, pH 4.0 to 5.4; morpholineethanesulfonic acid (MES), pH 5.6 to 6.6; Tris, pH 7.0 to 9.0; and N-cyclohexyl-3-aminopropyl sulfonic acid (CAPS), pH 9.7 to 11.0. After addition of CaCl$_2$, the protein was incubated at room temperature for 10 min. To analyze the effect of protease inhibitors on the autocleavage activity of NopE1, aprotinin (10 mM), leupeptin (10 mM), 4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF) (10 mM), or pepstatin A (5 μM) was added to purified NopE1 in separate samples in 100 mM Tris buffer (pH 7.5). Then, CaCl$_2$ was added to a final concentration of 10 mM and incubation continued for at least 15 min at 25°C.

### Analysis of the oligomerization state of NopE1. Oligomerization was analyzed by blue native gel electrophoresis. Experiments were performed as described previously (22). In short, a 5 to 16% acrylamide gradient gel was loaded with 3 μg purified protein. Electrophoresis was started at 4°C and 30 V. After the samples reached the stacking gel, electrophoresis was continued at 15 V for about 15 h and then at 110 V for 4 h. Gels were stained with Coomassie brilliant blue.

RESULTS

The DUF1521 domain of NopE1 is the central element for self-cleavage. In a previous study, we showed that purified NopE1 is cleaved at two positions in the presence of calcium (31). In order to determine the domain that is essential for cleavage, truncated versions of the *nopE1* open reading frame were created and expressed as GST fusions (Fig. 1). After addition of CaCl$_2$ to the purified protein, fragmentation was analyzed by SDS-PAGE. Removal of 32 or 97 amino acids at the C-terminal end (1–452 and 1–387 variants) affected cleavage at the C-terminal site but not at the N-terminal site. Further shortening from the C-terminal end resulted in protein variants with strongly reduced cleavage activity at the N-terminal site also (1–252 variant) or no cleavage (1–246 variant and smaller). To delimit the functional domain further, N-terminal deletions were created. A removal of the first 314 amino acids (315–484 variant), which preserves the complete C-terminal DUF1521 domain, resulted in a cleavable variant. A removal of 9 additional amino acids (324–484 variant) strongly reduced cleavage, which was abolished with a variant starting at position 334 (334–484 variant). This indicates that the domain which is required for efficient cleavage comprises about 140 amino acids, which encompasses most of the DUF1521 domain. The deletion of the N-terminal DUF1521 domain did not affect the activity of the C-terminal DUF1521 domain and
vice versa, proving that both DUF1521 domains are able to function independently.

Native NopE1 forms oligomers. The calculated molecular mass of NopE1 is 51 kDa. To analyze the quaternary structure, analytical gel filtration was applied. The elution profile (data not shown) indicated that the protein oligomerizes. For further analysis, blue native gel electrophoresis was applied (Fig. 2). The position of the dominant protein band suggests that NopE1 forms a dimer, and only a minor amount of the putative monomer was detected. The calcium-treated NopE1 was separated into three different bands. The dominant band had a mobility that is only slightly higher than that of the putative dimer of the full-length protein, suggesting that fragments stick together after cleavage.

For cleavage, a negatively charged amino acid next to the cleavage site and an aspartic acid residue in a predicted EF-hand motif are essential. A change of D147 to alanine blocks cleavage (31). To obtain more information about the possible self-cleavage mechanism, the aspartate was exchanged with asparagine or glutamate. Self-cleavage in the presence of calcium was observed for the D147E variant but not for the D147N variant (Fig. 3). This suggests an essential role of a negatively charged amino acid at this position.

Close to each cleavage site in NopE1, EF-hand-like motifs are present (Table 2). Plasmid pBD248 encodes a truncated NopE1 protein, which contains only the C-terminal motif (GST-NopE1135–484). In order to test if this motif is required for cleavage, the three aspartic acid residues (D365, D367, D387) were exchanged with alanine. The resulting truncated protein was purified and incubated without or with calcium for 10 min at room temperature. The cleavage of the proteins was analyzed by SDS-PAGE. Full-length cleavage products (fragments a, b, and c) are indicated by filled arrowheads. Truncated cleavage products are marked by dots. Fragments resulting from partial cleavage (fragment b+c) are marked by open arrowheads. In the 315–484 and 324–484 protein variants, the truncated fragment a shows an aberrant mobility.

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**FIG. 1.** The DUF1521 domain is required for self-cleavage. (A) GST-NopE1 and its truncated variants. The GST fusion partner is shown as a gray rectangle. Numbers at the left border indicate the amino acid range of NopE1 that is present in the fusion. Within the wild-type protein (1–484), cleavage takes place at two sites, D147 and D359 (filled triangles), which are located in two conserved domains of unknown function of DUF1521 (hatched rectangles). N-terminal truncations of NopE1 are depicted by dotted lines. The observed cleavage activities at the two sites are indicated by the following signs: +, cleavage as in the wild-type protein; +/−, most of the protein is not cleaved at the corresponding site; −, the protein is not cleaved. (B) Calculated molecular masses of hypothetical cleavage products of GST-NopE1 variants. a, b, and c correspond to the fragments depicted at the bottom of panel A. (C) GST-NopE1 and its truncated variants were purified and incubated without (−) or with (+) calcium for 10 min at room temperature. The cleavage of the proteins was analyzed by SDS-PAGE. Full-length cleavage products (fragments a, b, and c) are indicated by filled arrowheads. Truncated cleavage products are marked by dots. Fragments resulting from partial cleavage (fragment b+c) are marked by open arrowheads. In the 315–484 and 324–484 protein variants, the truncated fragment a shows an aberrant mobility.

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**TABLE 2.** Cleavage of GST-NopE1 variants without or with calcium.

<table>
<thead>
<tr>
<th>Protein variant</th>
<th>a (kDa)</th>
<th>b (kDa)</th>
<th>c (kDa)</th>
<th>Full length protein (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–484</td>
<td>41.8</td>
<td>22.3</td>
<td>13.4</td>
<td>77.5</td>
</tr>
<tr>
<td>1–452</td>
<td>41.8</td>
<td>22.3</td>
<td>10.0</td>
<td>74.1</td>
</tr>
<tr>
<td>1–387</td>
<td>41.8</td>
<td>22.3</td>
<td>3.3*</td>
<td>67.4</td>
</tr>
<tr>
<td>1–357</td>
<td>41.8</td>
<td>22.3</td>
<td>-</td>
<td>64.1</td>
</tr>
<tr>
<td>1–282</td>
<td>41.8</td>
<td>14.2</td>
<td>-</td>
<td>56.0</td>
</tr>
<tr>
<td>1–255</td>
<td>41.8</td>
<td>11.5</td>
<td>-</td>
<td>53.3</td>
</tr>
<tr>
<td>1–252</td>
<td>41.8</td>
<td>11.2</td>
<td>-</td>
<td>53.0</td>
</tr>
<tr>
<td>1–246</td>
<td>41.8*</td>
<td>10.6*</td>
<td>-</td>
<td>52.4</td>
</tr>
<tr>
<td>1–243</td>
<td>41.8*</td>
<td>10.3*</td>
<td>-</td>
<td>52.1</td>
</tr>
<tr>
<td>1–240</td>
<td>41.8*</td>
<td>10.0*</td>
<td>-</td>
<td>51.8</td>
</tr>
<tr>
<td>315–484</td>
<td>31.6</td>
<td>-</td>
<td>13.4</td>
<td>45.0</td>
</tr>
<tr>
<td>324–484</td>
<td>30.5</td>
<td>-</td>
<td>13.4</td>
<td>43.9</td>
</tr>
<tr>
<td>334–484</td>
<td>29.4*</td>
<td>-</td>
<td>13.4*</td>
<td>42.8</td>
</tr>
</tbody>
</table>

*fragments were not observed
and D371) were exchanged with alanine. Only the amino acid substitution D367A led to a noncleavable NopE1 variant (Fig. 4).

NopE1 cleavage shows broad temperature and pH-range stability. Purified NopE1 was incubated at temperatures up to 95°C for 5 min. Prewarmed calcium solution was added, and the calculated molecular mass of the fragments after cleavage are 22.3, 15.7, and 13.4 kDa. The aberrant positions of the bands suggest an oligomerization of cleaved and uncleaved protein. M, high-molecular-weight marker.

NopE1 cleavage shows broad temperature and pH-range stability. Purified NopE1 was incubated at temperatures up to 95°C for 5 min. Prewarmed calcium solution was added, and the calculated molecular mass of the fragments after cleavage are 22.3, 15.7, and 13.4 kDa. The aberrant positions of the bands suggest an oligomerization of cleaved and uncleaved protein. M, high-molecular-weight marker.

NopE1 cleavage is induced by calcium only and is not affected by protease inhibitors except for EDTA and EGTA. To determine the metal ion requirement of the autocleavage reaction, the purified protein was incubated in the presence of CaCl₂, CdCl₂, CoCl₂, CuCl₂, MgCl₂, MnCl₂, MnSO₄, NiCl₂, or ZnSO₄ in separate samples. Only calcium was able to induce self-cleavage of NopE1 (data not shown). Cleavage was not influenced by the protease inhibitors apronin, leupeptin, AEBSF, or pepstatin A but was inhibited by EDTA or EGTA (data not shown), indicating that a protease function is not involved in cleavage.

**DISCUSSION**

NopE1 is processed in the presence of calcium at two aspartate-proline bonds in the consensus GDPH motif, generated after heat treatment, NopE1 was incubated for 20 min at elevated temperatures (75 to 95°C) and subsequently cooled down to room temperature (0.1°C/5 s). Self-cleavage in the presence of calcium was observed for samples, which were previously incubated for 20 min up to 90°C (data not shown). In order to analyze the self-cleavage of NopE1 at different pHs, the protein was incubated in buffers from pH 4.0 to 11.0. In the presence of calcium, fragmentation of the protein was observed mainly between pH 4.0 and 9.7 and was most efficient between pH 7.0 and 8.5 (Fig. 6).

**TABLE 2. Cleavage sites and EF-hand-like motifs in NopE1**

<table>
<thead>
<tr>
<th>Motif</th>
<th>Sequence</th>
<th>Amino acid position in NopE1</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF-hand consensus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DKDGDPHTDFEE</td>
<td></td>
</tr>
<tr>
<td>N-terminal motif&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GD’PHV DGDGKPDPf</td>
<td>146–162</td>
</tr>
<tr>
<td>C-terminal motif&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GD’PHV DGDGKVDPDf</td>
<td>358–374</td>
</tr>
</tbody>
</table>

<sup>a</sup> EF-hand consensus sequence (6). Amino acid positions which are involved in coordination of calcium ions in characterized binding sites are underlined.

<sup>b</sup> EF-hand-like motif in the DUF1521 domain of NopE1. Cleavage site is indicated by a prime. Amino acid residues identical with the EF-hand consensus sequence are given in bold. Residues which are frequently observed in characterized EF-hand motifs are in capital letters; residues that are not observed in other EF-hand motifs are in lowercase.

![FIG. 2. Mobility of NopE1 in blue native acrylamide gel electrophoresis. Purified protein was loaded before (lane marked with ‘−’) or after (lane marked with ‘+’) treatment with calcium chloride. Bands of uncleaved NopE1 are marked with asterisks; filled arrowheads indicate the fragments of NopE1 after cleavage. The calculated molecular mass of the monomer is 51 kDa, and the calculated molecular masses of the fragments after cleavage are 22.3, 15.7, and 13.4 kDa.](http://jb.asm.org/)

![FIG. 3. Fragmentation of NopE1 with an amino acid exchange at the cleavage site. (A) GST-NopE1 cleavage sites and fragments (a, b, and c) and their predicted molecular masses. (B) The GST-NopE1 variants with D147N and D147E substitutions (encoded by plasmids pBJD216D147N and pBJD216D147E) were analyzed by SDS-PAGE after incubation with (+) or without (−) calcium chloride for 10 min at room temperature. Uncleaved proteins are marked with asterisks; fragments resulting from cleavage at both cleavage sites are marked with filled arrowheads; a fragment resulting from cleavage at the D359 cleavage site only is marked with an open arrowhead.](http://jb.asm.org/)

![FIG. 4. Effect of a site-specific amino acid exchange at the C-terminal EF-hand-like motif. NopE1*, N-terminally truncated NopE1 variant fused to GST (encoded by pBJD248); D367A, D367A, and D371A, variants of NopE1* with the corresponding amino acid exchange (encoded by pBJD248D367A, pBJD248D371A, and pBJD248D371A). Purified proteins were analyzed by SDS-PAGE after incubation with (+) or without (−) calcium chloride. N-terminal (31.6 kDa) and C-terminal (13.4 kDa) cleavage products are indicated. Fragment N shows an aberrant mobility.](http://jb.asm.org/)
bacillus pleuropneumoniae (18). In most cases, the two amino
blocked in the NopE1 variant D147N but not in the D147E
described previously (26). Surprisingly, the cleavage was
than the spontaneous hydrolysis of aspartate-proline bonds
the presence of calcium is several orders of magnitude faster
several weeks (data not shown), and the cleavage reaction in
18, 19, 28, 30). However, NopE1 is stable over a period of
ition under acidic conditions or during the aging of proteins (3,
ously proposed and explains hydrolysis after extended incuba-
15, 18, 28, 31, 33). A mechanism for cleavage of an aspartate
and FrpC can be processed at pHs between pH 5 and pH 9 (2,
processed at mild acidic and physiological pHs, both NopE1
proteins (16, 33), in zonadhesin (2), and in the prokaryotic
proteins pro-H3, zonadhesin, hemojuvelin, and MUC5AC are
share no significant sequence similarity with NopE1. While the
acids are part of a GDPH motif. Apart from that, the proteins
inhibitor pro-H3 (28), in several repulsive guidance molecule
ments comigrate. Purified NopE1 (51 kDa) was incubated with
FIG. 5. NopE1 is cleaved at moderate and elevated tempera-
tures. Purified NopE1 (51 kDa) was incubated with (+) or without
(−) calcium chloride for 30 min at the indicated temperatures.
Fragmentation was analyzed by SDS-PAGE. Above 70°C, the pro-
tein was no longer cleaved and precipitated in the presence of
calcium chloride. The full-length protein is 51 kDa in mass (+). A
37-kDa protein is due to partial fragmentation and is marked with
an open arrowhead. Filled arrowheads indicate the positions of
products from complete cleavage. The 15.7-kDa and 13.4-kDa frag-
ments comigrate.

ing three peptide fragments of 22.3 kDa, 15.7 kDa, and 13.4
kDa in size (31). The cleavage of a peptide bond between
aspartate and proline was described for several proteins: in the
eukaryotic mucin proteins, MUC2 (14) and MUC5AC (15), in
the rat sialomucin complex (25), in the precursor of pre-α-
inhibitor pro-H3 (28), in several repulsive guidance molecule
proteins (16, 33), in zonadhesin (2), and in the prokaryotic
proteins FrpC of Neisseria meningitidis and ApxIVA of Actino-
bacillus pleuropneumoniae (18). In most cases, the two amino
acids are part of a GDPH motif. Apart from that, the proteins
share no significant sequence similarity with NopE1. While the
proteins pro-H3, zonadhesin, hemojuvelin, and MUC5AC are
processed at mild acidic and physiological pHs, both NopE1
and FrpC can be processed at pHs between pH 5 and pH 9 (2,
15, 18, 28, 31, 33). A mechanism for cleavage of an aspartate
(or asparagine)-proline bond, which is based on the intramole-
cular reaction between the aspartate (or asparagine) side
chain and the adjacent peptide bond with proline, was previ-
ously proposed and explains hydrolysis after extended incuba-
tion under acidic conditions or during the aging of proteins (3,
18, 19, 28, 30). However, NopE1 is stable over a period of
several weeks (data not shown), and the cleavage reaction in
the presence of calcium is several orders of magnitude faster
than the spontaneous hydrolysis of aspartate-proline bonds
described previously (26). Surprisingly, the cleavage was
blocked in the NopE1 variant D147N but not in the D147E
variant (Fig. 3), indicating that the carboxylic acid function of
the side chain is essential for cleavage.

To our knowledge, induction of the aspartate-proline bond
cleavage by calcium was observed only for NopE1, NopE2,
FrpC, and ApxIVA (18, 31). These proteins possess EF-hand-
like motifs (Table 2), which are indicative for calcium binding
and which might be required for cleavage. This is supported by
the finding that the exchange of a conserved aspartic acid
residue within this motif prevented cleavage. Most likely, the
binding of calcium ions results in conformational changes,
which lead to the activation of a nonprotease cleavage mech-
anism. This is in agreement with the observation that self-
cleavage proceeded in the presence of various protease inhibi-
tors but not in the presence of an excess of the metal ion
chelators EDTA and EGTA (31).

Interestingly, besides calcium no other tested metal ion, e.g.,
manganese or magnesium, induced cleavage. Calcium is a well-
known signaling molecule in the symbiotic interaction (17).
Manganese is an essential micronutrient (20, 21); magnes-
iunm is the most abundant divalent metal cation in the plant
cytosol (24). Therefore, the strict selectivity might be a
biological necessity to prevent an ill-timed cleavage within
the plant cell.

The self-cleavage capability of the type III-secreted effector
NopE1 is essential for its biological activity (31). The two cleavage
sites are located within a duplicated domain (DUF1521). Here
we showed that this domain is the central functional unit (Fig.
1). Both domains are also cleaved if the other domain is ab-
sent, speaking against the requirement of having two similar
domains within one protein. However, this does not exclude
that intramolecular or intermolecular interactions occur. In
support of this, we found that the protein as well as cleavage
products oligomerize (Fig. 2). We do not know if oligomeriza-
tion is the prerequisite for cleavage. It is of interest that a few
predicted proteins with significant similarity to NopE1 exist
(Fig. 7). They all contain a single DUF1521 domain. No ex-
perimental data concerning these proteins exist. However,
bioinformatics analyses indicate that at least two of the corre-
sponding bacteria (Burkholderia phytofirmans PsNJ and Vibrio
corallilyticus ATCC BAA450) are likely to encode a type III
secretion system. B. phytofirmans, a betaproteobacterium, is a
plant-beneficial bacterium which was first isolated from on-
ion roots (4, 23). V. corallilyticus, a gammaproteobacterium,
causes tissue lysis of the coral Pocillopora damicornis (1). It
seems that the DUF1521 domain is more widespread in
proteobacteria and might contribute to the interaction with host organisms.

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REFERENCES