Crystallization and preliminary X-ray analysis of
two variants of the *Escherichia coli* O157
ParE2–PaaA2 toxin–antitoxin complexYann G. J. Sterckx,^{a,b} Sarah
Haesaerts,^{a,b} Laurence Van
Melderen^c and Remy Loris^{a,b*}^aStructural Biology Brussels, Department of
Biotechnology, Vrije Universiteit Brussel,
Pleinlaan 2, B-1050 Brussel, Belgium,^bStructural Biology Research Center, VIB,
Pleinlaan 2, B-1050 Brussel, Belgium, and^cGénétique et Physiologie Bactérienne, IBMM,
Université Libre de Bruxelles (ULB), 12 Rue des
Professeurs Jeener et Brachet, B-6041 Gosselies,
Belgium

Correspondence e-mail: remy.loris@vib-vub.be

Received 6 June 2014

Accepted 30 July 2014

The *paaR2–paaA2–parE2* operon is a three-component toxin–antitoxin module encoded in the genome of the human pathogen *Escherichia coli* O157. The toxin (ParE2) and antitoxin (PaaA2) interact to form a nontoxic toxin–antitoxin complex. In this paper, the crystallization and preliminary characterization of two variants of the ParE2–PaaA2 toxin–antitoxin complex are described. Selenomethionine-derivative crystals of the full-length ParE2–PaaA2 toxin–antitoxin complex diffracted to 2.8 Å resolution and belonged to space group $P4_12_12$ (or $P4_32_12$), with unit-cell parameters $a = b = 90.5$, $c = 412.3$ Å. It was previously reported that the full-length ParE2–PaaA2 toxin–antitoxin complex forms a higher-order oligomer. In contrast, ParE2 and PaaA2_{13–63}, a truncated form of PaaA2 in which the first 12 N-terminal residues of the antitoxin have been deleted, form a heterodimer as shown by analytical gel filtration, dynamic light scattering and small-angle X-ray scattering. Crystals of the PaaA2_{13–63}–ParE2 complex diffracted to 2.7 Å resolution and belonged to space group $P6_122$ (or $P6_522$), with unit-cell parameters $a = b = 91.6$, $c = 185.6$ Å.

1. Introduction

Toxin–antitoxin (TA) modules constitute a stress-response network that provides bacteria and archaea with tools enabling swift adaptation to ever-changing environments. These modules are widespread in the genomes of bacteria and archaea (Leplae *et al.*, 2011; Blower *et al.*, 2012) and have been linked to the adaptation of the metabolism of the cell under physiological stress (Christensen *et al.*, 2003; Cooper *et al.*, 2009; Christensen-Dalsgaard *et al.*, 2010), the generation of persister cells (Shah *et al.*, 2006; Maisonneuve *et al.*, 2011), quorum-sensing-induced and stress-induced programmed cell death (Sat *et al.*, 2003; Hazan *et al.*, 2004; Kolodkin-Gal *et al.*, 2007) and the maintenance of unstable genetic elements carrying genes necessary for survival of a bacterium (Moritz & Hergenrother, 2007; Szekeres *et al.*, 2007). Typically, TA modules are two-gene operons comprising the open reading frame of the toxin preceded by the gene encoding the antitoxin, which can either be an mRNA or a protein molecule (Gerdes *et al.*, 2005; Blower *et al.*, 2011). Although this constitutes the archetypal TA module, variations on this theme have been reported (Christensen-Dalsgaard & Gerdes, 2006; Hallez *et al.*, 2010). In type II TA modules, which are the most frequent, both the toxin and the antitoxin are proteins. TA toxins have the ability to completely shut down the metabolism of the cell by interacting with key components of the cellular machinery. Known targets for TA-encoded toxins are DNA gyrase (Bernard & Couturier, 1992; Jiang *et al.*, 2002; Dao-Thi *et al.*, 2005; Yuan *et al.*, 2010), elongation factor Ef-Tu (Castro-Roa *et al.*, 2013), the ribosome (Vesper *et al.*, 2011; Zhang & Inouye, 2011; Schifano *et al.*, 2013), mRNA (Pedersen *et al.*, 2003; Zhang, Zhang & Inouye, 2003; Neubauer *et al.*, 2009), tRNA (Winther & Gerdes, 2011), components for cell-wall synthesis (Mutschler *et al.*, 2011) and glutamyl-tRNA synthetase GltX (Germain *et al.*, 2013). Luckily for the cell, the activity of the toxin can be countered by its cognate antitoxin (Pedersen *et al.*, 2002; Zhang, Zhang, Hoefflich *et al.*, 2003; Zhao & Zhang, 2008). These activities are regulated *via* the antitoxin at the levels of both protein activity and transcription (for a review, see Loris & Garcia-Pino, 2014).

Type II TA toxins and antitoxins are categorized into toxin and antitoxin superfamilies, respectively. The toxin superfamilies are defined based on the three-dimensional structures of the toxins. To date, seven toxin superfamilies (MazF/CcdB, RelE/ParE, VapC, HipA, Doc, ζ and HicA) have been identified. However, this number is expected to increase, as type II toxins unrelated to the mentioned superfamilies have been discovered through combined computational and experimental approaches (Florek *et al.*, 2011; Leplae *et al.*, 2011; Russell *et al.*, 2011; Smith *et al.*, 2011; Ren *et al.*, 2012). The diversity in antitoxin superfamilies exceeds that of the toxins as 20 antitoxin superfamilies have already been listed (Leplae *et al.*, 2011): Phd, RelB, PasA, MazE, HigA, VapX, CcdA, ε , ParD and Fiza–FizK. The archetypal type II antitoxins are dimers consisting of two domains with distinct functions. The N-terminal part is a DNA-binding domain (DBD), which varies from superfamily to superfamily. The C-terminal moiety serves as a toxin-binding domain and is intrinsically disordered in most antitoxins when not in complex with the toxin (Madl *et al.*, 2006; Oberer *et al.*, 2007; Garcia-Pino *et al.*, 2010). Upon binding of the antitoxin to its cognate toxin, this part wraps itself around the toxin, thereby adopting structure in a process called ‘folding upon binding’ (Kamada & Hanaoka, 2005; Garcia-Pino *et al.*, 2008; De Jonge *et al.*, 2009). However, the antitoxin superfamilies also contain atypical variants which harbour features that deviate from the aforementioned hallmarks. These antitoxins can be (i) dimers in which the roles of the termini have been inverted (Brown *et al.*, 2009),

(ii) dimers without DBDs (Meinhart *et al.*, 2003) or (iii) monomers without DBDs (Takagi *et al.*, 2005).

As an additional variation on the type II TA modules, three-component systems have been described in which the antitoxin is split into a DNA-binding regulator and a non-DNA-binding antitoxin. The prophage region of the *Escherichia coli* O157 genome harbours two such three-component type II TA modules: *paaR1–paaA1–parE1* and *paaR2–paaA2–parE2* (Hallez *et al.*, 2010). The toxins, ParE1 and ParE2, are members of the RelE/ParE toxin superfamily. *In vivo*, expression of the *parE* genes leads to cell death by eliciting the SOS response, an event which can be countered by the respective antitoxins, PaaA1 and PaaA2 (Hallez *et al.*, 2010). These antitoxins are atypical members of the RelB antitoxin superfamily as they contain no DBD and exist as monomers in solution (Sterckx *et al.*, 2014). Transcriptional repression of both operons is mainly ensured by the respective regulators, PaaR1 and PaaR2, which are encoded upstream of the antitoxin and toxin genes and contain a DNA-binding domain of the DicA transcriptional repressor family. It is clear that PaaA2 and ParE2 physically interact with each other as previous reports have shown that the proteins co-purify and form a large oligomeric complex in solution, which was proposed to be a heterododecamer (Sterckx *et al.*, 2012). To date, it has not been shown that PaaR2 physically interacts with the ParE2–PaaA2 toxin–antitoxin complex (Hallez *et al.*, 2010). Previously reported crystals of full-length ParE2–PaaA2 only diffracted to about 4 Å resolution and

pET21b-A2-E2His

FLAG	
ATG GAT TAT AAA GAT GAC GAT GAC AAA	AAT AGA GCC CTT TCA CCA ATG GTT TCT GAA TTT GAA ACC ATT GAA
M D Y K D D D D K N R A L S P M V S E F E T I E	
CAA GAA AAC AGT TAC AAC GAA TGG CTG CGT GCG AAA GTA GCA ACG AGC CTT GCA GAT CCG CGC CCA GCA ATT	
Q E N S Y N E W L R A K V A T S L A D P R P A I	
CCG CAT GAC GAA GTT GAG CGC AGA ATG GCA GAA CGC TTT GCT AAG ATG CGC AAG GAA CGG AGC AAG CAG TAA	
P H D E V E R R M A E R F A K M R K E R S K Q *	
A ATG TTA CCC GTG TTA TGG CTT GAA AGC GCA GAT ACC GAC CTA GAT GAT ATA ACT AGT TAT ATT GCT CGT	
M L P V L W L E S A D T D L D D I T S Y I A R	
TTC GAC ATA GAT GCG GCT GAA CGC TTA TGG CAG CGA TTA AGG GGT TGT GTG CTG CCG TTA TCC GAA CAT CCG	
F D I D A A E R L W Q R L R G C V L P L S E H P	
TAT TTA TAC CCA CCA AGC GAC AGA GTA CCT GGC TTG CGT GAG ATT GTA GCC CAC CCT AAC TAT ATA ATT CTA	
Y L Y P P S D R V P G L R E I V A H P N Y I I L	
TAC CGC GTA ACA ACA TCA AGC GTT GAA GTA GTA AAC GTG ATC CAC GCA AGA CGC CAG TTT CCC	CTC GAG CAT
Y R V T T S S V E V V N V I H A R R Q F P L E H	
CAT CAT CAT CAT CAT TAA	
H H H H H *	

PaaA2 = 8.46 kDa
ParE2 = 11.73 kDa

pET21b-A2_{13–63}-HisE2

ATG GAA ACC ATT GAA CAA GAA AAC AGT TAC AAC GAA TGG CTG CGT GCG AAA GTA GCA ACG AGC CTT GCA GAT	
M E T I E Q E N S Y N E W L R A K V A T S L A D	
CCG CGC CCA GCA ATT CCC CAT GAC GAA GTT GAG CGC AGA ATG GCA GAA CGC TTT GCT AAG ATG CGC AAG GAA	
P R P A I P H D E V E R R M A E R F A K M R K E	
CGG AGC AAG CAG TAA	A ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC
R S K Q *	M G S S H H H H H H S S G L V P R G
AGC CAT TTA CCC GTG TTA TGG CTT GAA AGC GCA GAT ACC GAC CTA GAT GAT ATA ACT AGT TAT ATT GCT CGT	
S H L P V L W L E S A D T D L D D I T S Y I A R	
TTC GAC ATA GAT GCG GCT GAA CGC TTA TGG CAG CGA TTA AGG GGT TGT GTG CTG CCG TTA TCC GAA CAT CCG	
F D I D A A E R L W Q R L R G C V L P L S E H P	
TAT TTA TAC CCA CCA AGC GAC AGA GTA CCT GGC TTG CGT GAG ATT GTA GCC CAC CCT AAC TAT ATA ATT CTA	
Y L Y P P S D R V P G L R E I V A H P N Y I I L	
TAC CGC GTA ACA ACA TCA AGC GTT GAA GTA GTA AAC GTG ATC CAC GCA AGA CGC CAG TTT CCC TAA	
Y R V T T S S V E V V N V I H A R R Q F P *	

PaaA2_{13–63} = 6.23 kDa
ParE2 = 12.70 kDa

Figure 1

Coding regions of the used expression vectors. The ORFs of *paaA2* and *parE2* are highlighted by blue and red boxes, respectively. Grey boxes indicate purification tags and protease cleavage sites. The single nucleotide separating the *paaA2* and *parE2* ORFs is indicated in bold. The resulting amino-acid sequences are also shown in bold and the molecular masses of the produced proteins are indicated.

did not allow structure determination (Sterckx *et al.*, 2012). In this paper, we report a new crystal form of the ParE2–PaaA2 toxin–antitoxin complex. Furthermore, we also describe the crystallization and hydrodynamic characterization of the complex between ParE2 and PaaA2_{13–63}, a PaaA2 truncation in which the first 12 N-terminal residues of the antitoxin have been deleted.

2. Material and methods

2.1. Cloning, protein production and purification

Two expression vectors, pET21b-A2-E2His and pET21b-A2_{13–63}-HisE2, were constructed and encode the full-length and truncated ParE2–PaaA2 complexes, respectively. pET21b-A2-E2His was obtained by extracting the *paaA2*–*parE2* coding region (UniProtKB accession Q8XAD5 and Q8X366) from *E. coli* O157:H7 EDL933 as described by Hallez *et al.* (2010) and introducing it into the pET-21b vector (Novagen) via the *EcoRI* and *XhoI* sites using the primers 5'-GAATTCAGGAGGGAGTAATGGATTATAAAGATGACGATG-ACAAAATAGAGCCCTTTCACCA-3' (forward) and 5'-CGCA-AGACGCCAGTTTCCCTCGAG-3' (reverse). This construct provides PaaA2 with an N-terminal FLAG tag (DYKDDDDK) and ParE2 with a C-terminal His tag (LEHHHHHH). pET21b-A2_{13–63}-HisE2 was created by modifying pET21b-A2-E2His through commercial cloning (GenScript). Briefly, the region encoding the FLAG tag and the first 12 native residues of PaaA2 were deleted, and the His tag of ParE2 was displaced from the C-terminus to the N-terminus and equipped with a thrombin cleavage site. The resulting vector thus carries a truncated version of PaaA2 (PaaA2_{13–63}) and equips ParE2 with an N-terminal His tag with a thrombin cleavage site (MGSSHHHHHHSSGLVPRGSH). Transformation of the expression vectors and protein production and purification were performed as described by Sterckx *et al.* (2012). The progress of the purification was monitored by SDS–PAGE analysis. The samples were loaded onto a 15% acrylamide gel (30% acryl/bisacrylamide, 1.5 M Tris pH 8.0, 10% SDS, 10% APS, 1% TEMED). Electrophoresis was performed in 1× Laemmli buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS) for 20 min at 180 V, 20 min at 190 V and 20 min at 200 V. Protein bands were visualized with Coomassie Brilliant Blue R250 and molecular masses were determined using the Prestained Protein Molecular Weight Marker (Fermentas). The amino-acid sequences of the proteins encoded by pET21b-A2-E2His and pET21b-A2_{13–63}-HisE2 are shown in Fig. 1.

In order to obtain an SeMet derivative of the full-length ParE2–PaaA2 complex, pET21b-A2-E2His was purified from *E. coli* strain BL21 (DE3) using a Qiagen Miniprep according to the manufacturer's instructions. The purified vector was transformed into the auxotrophic *E. coli* strain B834 (DE3) (Studier *et al.*, 1990) using the CaCl₂ method (Hanahan *et al.*, 1991). Transformed cells were selected on LB plates containing 100 µg ml^{−1} ampicillin and 2% (w/v) glucose and were grown overnight at 310 K. A single colony was re-plated on a minimal medium plate supplemented with 100 µg ml^{−1} ampicillin, 0.2% (w/v) glucose and 50 µg ml^{−1} L-Met. Expression was first tested on a small scale in SeMet medium (Molecular Dimensions). An individual colony was picked to start an overnight 5 ml pre-culture incubated in SeMet medium supplemented with 100 µg ml^{−1} ampicillin, 0.2% (w/v) glucose and 50 µg ml^{−1} L-Met at 310 K with aeration. After overnight incubation, the cells were pelleted and washed three times with 5 ml sterile water before being resuspended in 1 ml sterile water. A main culture was then started by inoculating an Erlenmeyer flask containing 50 ml pre-warmed (310 K) SeMet medium supplemented with 100 µg ml^{−1} ampicillin, 0.2% (w/v)

glucose and 50 µg ml^{−1} L-SeMet with the 1 ml pre-culture. Expression of the genes was induced by the addition of 1 mM IPTG after a suitable OD₆₀₀ of 0.6–0.8 had been reached. For large-scale expression, four 50 ml SeMet medium pre-cultures supplemented with 100 µg ml^{−1} ampicillin, 0.2% (w/v) glucose and 50 µg ml^{−1} L-Met were started. After overnight incubation, the cells were pelleted and washed three times with 50 ml sterile water before being resuspended in 1 ml sterile water. A main culture of 4 l was then started by inoculating flasks containing 1 l pre-warmed (310 K) SeMet medium supplemented with 100 µg ml^{−1} ampicillin, 0.2% (w/v) glucose and 50 µg ml^{−1} L-SeMet with the 1 ml pre-cultures. Expression of the genes was induced by the addition of 1 mM IPTG after a suitable OD₆₀₀ of 0.6–0.8 had been reached. Cultures were grown overnight at 310 K with aeration and were harvested by centrifugation for 20 min [6500g (5000 rev min^{−1} in a JLA-8.1000 rotor), 277 K]. The bacterial pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 0.1 mg ml^{−1} AEBSF, 1 µg ml^{−1} leupeptin, 1 mM EDTA) and aliquoted in volumes of 50 ml. The aliquots were cryo-quenched using liquid nitrogen and stored at 192 K. The SeMet derivative of the full-length ParE2–PaaA2 complex was purified as described by Sterckx *et al.* (2012). The identities of SeMet-derivatized PaaA2 and ParE2 were confirmed by N-terminal sequencing (Alta Biosciences).

2.2. Crystallization

Protein samples were dialyzed against a suitable buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl) and concentrated to 5–20 mg ml^{−1} (Amicon Ultra UltraCel 3K). Crystallization conditions were screened manually using the hanging-drop vapour-diffusion method in 48-well plates (Hampton VDX greased) with drops consisting of 2 µl protein solution (between 7 and 10 mg ml^{−1}) and 2 µl reservoir solution equilibrated against 125 µl reservoir solution. Commercial screens from Hampton Research (Crystal Screen, Crystal Screen 2, Natrix and Natrix 2), Molecular Dimensions (Morpheus, ProPlex 1 and ProPlex 2), and Jena Bioscience (JBScreen Classic 1–4) were used for initial screening. The FLAG and His tags were retained on the proteins for crystallization.

For the full-length ParE2–PaaA2 complex, crystals were obtained through variation of the precipitant concentration [4–16% (w/v) PEG 6000], protein concentration (5–15 mg ml^{−1}) and pH (5.0–7.0) of a promising hit [Molecular Dimensions ProPlex 1 condition No. 41: 100 mM MgCl₂, 100 mM MES pH 6.0, 8% (w/v) PEG 6000] in an attempt to further optimize this condition. The final crystallization condition differed by only 0.1 pH unit from the initial hit [100 mM MgCl₂, 100 mM MES pH 6.1, 8% (w/v) PEG 6000] and yielded plate-like crystals with a reasonable thickness. Diffraction-quality crystals typically grew after approximately 7 d at 293 K using protein concentrations between 7 and 11.5 mg ml^{−1}. Crystals of the SeMet derivative of the full-length ParE2–PaaA2 complex were obtained by micro-seeding with the native complex. Briefly, native crystals were transferred to 50 µl mother liquor [100 mM MgCl₂, 100 mM MES pH 6.1, 8% (w/v) PEG 6000] and vortexed (15 s) to obtain microseeds, which were used immediately. Serial dilutions of the seeds ranging from 1 to 10^{−7} were mixed in a 1:1 ratio (2 µl each) with fresh SeMet-derivatized protein solution at 10 mg ml^{−1} and equilibrated against the mother liquor. Diffraction-quality crystals typically grew after approximately 7 d at 293 K.

For ParE2–PaaA2_{13–63}, a single large crystal of up to 300 µm in length grew in Molecular Dimensions ProPlex 2 condition No. 20 (100 mM sodium acetate pH 5.0, 1 M ammonium sulfate) about a month after the screen had been set up. The protein concentration used was 11.5 mg ml^{−1} and the crystal plate was incubated at 293 K.

2.3. Data collection and processing

All data sets were collected on the PROXIMA1 beamline at the SOLEIL synchrotron (Gif-Sur-Yvette, France) and were processed with *XDS* (Kabsch, 2010). The quality of the collected data sets was verified by close inspection of the *XDS* output files and through *phenix.xtriage* (Zwart *et al.*, 2005) in the *PHENIX* package (Adams *et al.*, 2010). Twinning tests were also performed by *phenix.xtriage*. Analysis of the unit-cell contents was performed with the program *MATTHEWS_COEF*, which is part of the *CCP4* package (Winn *et al.*, 2011).

Crystals of the SeMet derivative of the full-length ParE2–PaaA2 complex were cryo-quenched in liquid nitrogen with the addition of 15% (v/v) glycerol to the mother liquor as a cryoprotectant. For the final data collection, 400° of data were collected from a single SeMet-derivative crystal in 0.1° slices using a PILATUS 6M detector with an exposure time of 0.1 s deg^{−1}, a crystal-to-detector distance of 600.8 mm and a wavelength of 0.97911 Å.

The single ParE2–PaaA2_{13–63} crystal was cryo-quenched in liquid nitrogen with the addition of 15% (v/v) glycerol to the mother liquor as a cryoprotectant. For the final data collection, 200° of data were collected from a single crystal in 0.2° slices using a PILATUS 6M detector with an exposure time of 0.4 s deg^{−1}, a crystal-to-detector distance of 577.45 mm and a wavelength of 0.97911 Å.

2.4. Hydrodynamic characterization

Analytical gel-filtration experiments were performed on an ÄKTAexplorer platform (GE Healthcare) using a Superdex 75 HR 10/30 column (GE Healthcare) to determine the size and molecular mass of the purified ParE2–PaaA2_{13–63} complex. The column was pre-equilibrated using running buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl) for at least one column volume. A 500 µl sample at a concentration of 2 mg ml^{−1} was injected using running buffer with a flow rate of 0.5 ml min^{−1}. To determine the molecular masses and the hydrodynamic radii of the proteins (Uversky, 1993), the column was calibrated using molecular-mass standards from Bio-Rad.

Dynamic light-scattering (DLS) experiments were performed on a DynaPro plate reader (Wyatt Technology) in 50 mM Tris–HCl pH 8.0, 500 mM NaCl at room temperature (293 K). Data were measured for PaaA2_{13–63}–ParE2 samples at different concentrations (1–10 mg ml^{−1}).

Small-angle X-ray scattering (SAXS) studies were conducted on the SWING beamline at the SOLEIL synchrotron, Gif-sur-Yvette, France in HPLC mode (David & Pérez, 2009). A total volume of 80 µl ParE2–PaaA2_{13–63} sample at 12 mg ml^{−1} was injected onto a KW402.5-4F column (Shodex) which had been pre-equilibrated with running buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl) for at least one column volume. The flow rate was set to 0.25 ml min^{−1} and data were collected with an exposure time of 0.75 s and a dead time of 1.5 s. Buffer data were collected at the beginning of the chromatogram and sample data were collected in the peak area. Data processing and analysis was performed with the programs provided in the *ATSAS* package (Petoukhov *et al.*, 2012), the online *SAXSMoW* application (Fischer *et al.*, 2010) and the *Q_R* method (Rambo & Tainer, 2013).

3. Results and discussion

The crystallization of the full-length ParE2–PaaA2 toxin–antitoxin complex in space group *P*₃₁21 (or *P*₃₂21) has been described previously (Sterckx *et al.*, 2012). While crystals of the native complex could be obtained, they only diffracted to 3.8 Å resolution at best. In the absence of a suitable model for molecular replacement, efforts were directed towards obtaining heavy-atom and/or SeMet derivatives. Unfortunately, these attempts failed as the incorporation of heavy atoms reduced the diffraction power of the derivative crystals to a resolution of 8 Å in the best case. Further screening of crystallization conditions was pursued to find other diffraction-quality crystal forms. A promising hit was obtained from Molecular Dimensions ProPlex 1 condition No. 41 [100 mM MgCl₂, 100 mM MES pH 6.0, 8% (w/v) PEG 6000]. As can be seen from Fig. 2(a), the drop contains micro-crystals, which are unsuitable for diffraction experiments. Optimization of this initial hit led to a final crystallization condition [100 mM MgCl₂, 100 mM MES pH 6.1, 8% (w/v) PEG 6000] which differed by only 0.1 pH unit from the initial hit and yielded plate-like crystals with a reasonable thickness (Fig. 2b). Crystallization of the SeMet derivative of the ParE2–PaaA2 complex in this crystal form initially failed. SeMet-derivatized crystals could only be obtained through micro-seeding with seeds of the native crystals. Suitably sized SeMet-derivative crystals were produced by using a tenfold dilution of the native crystal seeds and incubating the

Figure 2 Morphology of native and of SeMet-derivatized crystals of the full-length ParE2–PaaA2 toxin–antitoxin complex. (a) Microcrystals of the native full-length ParE2–PaaA2 toxin–antitoxin complex obtained in 100 mM MgCl₂, 100 mM MES pH 6.0, 8% (w/v) PEG 6000. (b) Optimization of the initial hit shown in (a) led to a final crystallization condition in 100 mM MgCl₂, 100 mM MES pH 6.1, 8% (w/v) PEG 6000. Typical crystals appear after incubation for around 7 d at 293 K. (c) SeMet-derivatized crystals of the full-length ParE2–PaaA2 toxin–antitoxin complex prepared by micro-seeding with a 10^{−1} dilution of crushed fragments of native crystals similar to those in (b).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	SeMet-derivatized ParE2–PaaA2	ParE2–PaaA2 _{13–63}
Beamline	PROXIMA1, SOLEIL	PROXIMA1, SOLEIL
Wavelength (Å)	0.97911	0.97911
Resolution range (Å)	49.37–2.83 (2.93–2.83)	48.78–2.67 (2.77–2.67)
Unit-cell parameters (Å, °)	$a = b = 90.6$, $c = 412.3$, $\alpha = \beta = \gamma = 90$	$a = b = 91.6$, $c = 185.6$, $\alpha = \beta = 90$, $\gamma = 120$
Space group	$P4_12_12$ or $P4_32_12$	$P6_122$ or $P6_522$
Mosaicity (°)	0.077	0.076
$R_{\text{meas}}^\dagger$	0.14 (0.85)	0.19 (1.23)
No. of measured reflections	1086420 (90564)	280494 (23257)
No. of unique reflections	42331 (4079)	13723 (1333)
Multiplicity	25.7 (22.2)	20.4 (17.4)
$\langle I/\sigma(I) \rangle$	23.91 (3.57)	17.62 (2.50)
Completeness (%)	99.82 (98.17)	99.91 (99.33)
$\langle F(+) - F(-) \rangle / \sigma[F(+) - F(-)]$	1.532 (0.757)	
$R_{\text{anom}}(\%)^\ddagger$	5.2 (18.6)	
$R_{\text{p.i.m.}}(\%)^\S$	3.0 (17.6)	

$^\dagger R_{\text{meas}} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$ (Diederichs & Karplus, 1997). $^\ddagger R_{\text{anom}} = \sum_{hkl} |I(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \langle I(hkl) \rangle$ (Weiss, 2001). $^\S R_{\text{p.i.m.}} = \sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$ (Weiss & Hilgenfeld, 1997).

drops at 293 K (Fig. 2c). Prior to data collection, the SeMet-derivatized crystals were cryo-quenched in liquid nitrogen with an additional cryoprotectant in two different cryosolutions, either by increasing the PEG 6000 concentration to 30%(w/v) or by the addition of 15%(v/v) glycerol to the mother liquor. The presence of selenium in the cryo-quenched protein crystals was confirmed at the beamline by an X-ray fluorescence scan (Fig. 3). Interestingly, the crystals cryo-quenched in the first cryosolution had no diffraction power at all, while the crystals cryo-quenched in the cryosolution containing 15%(v/v) glycerol typically diffracted to a resolution of 2.8 Å (Fig. 4a). The crystals belonged to space group $P4_12_12$ (or $P4_32_12$), with unit-cell parameters $a = b = 90.5$, $c = 412.3$ Å (Table 1). This unit cell is consistent with a large oligomeric species as in the case of the $P3_121$ (or $P3_221$) crystal form of the ParE2–PaaA2 toxin–antitoxin complex described previously (Sterckx *et al.*, 2012). Data were also collected from the native crystals, which had the same diffraction quality. However, as will be discussed below, the single data set collected from the SeMet-derivatized crystals should prove sufficient to allow structure determination.

Figure 3
An X-ray fluorescence scan (relative units) of a crystal of an SeMet derivative of the full-length ParE2–PaaA2 complex displays an absorption peak close to 12.66 keV, which is typical for SeMet.

Recently, it was suggested that the first 12 N-terminal residues of PaaA2 could be important for oligomerization of the ParE2–PaaA2 toxin–antitoxin complex. Furthermore, based on sequence comparisons within the RelB antitoxin superfamily and the solution structure of the PaaA2 antitoxin, it was envisaged that these residues would not be crucial for toxin inhibition *in vivo* (Sterckx *et al.*, 2014). Indeed, the

Figure 4
(a) Diffraction pattern of an SeMet-derivatized crystal similar in size to that shown in Fig. 1(c). It shows diffraction spots to 2.8 Å resolution (the 2.8 Å resolution limit is indicated by an orange circle). (b) Diffraction pattern of the only crystal of the ParE2–PaaA2_{13–63} complex. The crystal shows diffraction spots to 2.7 Å resolution (the 2.7 Å resolution limit is indicated by an orange circle).

*paaA2*_{13–63} and *parE2* genes can be easily co-expressed in *E. coli* BL21 (DE3) cells without any toxicity effects on the bacterial culture. Assuming equimolar production of proteins ($\epsilon_{280\text{ nm}} = 1.34\text{ mg}^{-1}\text{ ml cm}^{-1}$ for a ParE2–PaaA2_{13–63} heterodimer), about 20 mg protein is produced per litre of culture after overnight induction. During IMAC, a single peak containing both proteins elutes at 0.15 M imidazole. A subsequent gel-filtration step to polish the protein preparation reveals that PaaA2_{13–63} and ParE2 co-elute in a single peak, suggesting the existence of a ParE2–PaaA2_{13–63} toxin–antitoxin complex. To characterize its hydrodynamic behaviour and its oligomerization state in solution, a combination of analytical gel filtration, DLS and SAXS was employed. As can be seen from Figs. 5(a) and 5(b), analytical gel-filtration and DLS experiments reveal that the ParE2–PaaA2_{13–63} complex has hydrodynamic properties corresponding to those expected for a heterodimer (theoretical molecular mass of 18.92 kDa). The higher values for the molecular mass and hydrodynamic radius obtained *via* DLS compared with

those from analytical gel filtration are most probably a consequence of the long N-terminal His tag on the toxin (see Fig. 1). The heterodimeric state of the ParE2–PaaA2_{13–63} complex is confirmed by the results of the SAXS analysis (Figs. 5c–5e), which show that the scattering particle is small ($R_g = 21.70\text{ Å}$; $D_{\text{max}} = 75.54\text{ Å}$) and has an estimated molecular mass of around 20 kDa (21.10 kDa as estimated by *SAXSMoW* and 18.62 kDa by the Q_R method), which matches the expected molecular mass very well. Although the complex behaves as a globular entity in solution, the normalized Kratky plot suggests the presence of flexibility in the scattering particle (Fig. 5d), which can again be accounted for by the flexibility of the long N-terminal His tag on the toxin.

In order to obtain diffraction-quality crystals of the ParE2–PaaA2_{13–63} complex, many different conditions were screened (variation in protein concentration, temperature and crystallization screens and optimization of the conditions thereof). However, none of them seemed to produce promising hits. About a month after the

Figure 5

The ParE2–PaaA2_{13–63} complex exists as a heterodimer in solution. (a) Analytical gel filtration. The experiment was performed on a Superdex 75 HR 10/30 column. The black and grey traces indicate the elution profiles for the ParE2–PaaA2_{13–63} complex and the Bio-Rad standard (A, bovine thyroglobulin, molecular mass 670 kDa, $R_h = 8.6\text{ nm}$; B, bovine γ -globulin, molecular mass 158 kDa, $R_h = 5.1\text{ nm}$; C, chicken ovalbumin, molecular mass 44 kDa, $R_h = 2.8\text{ nm}$; D, horse myoglobin, molecular mass 17 kDa, $R_h = 1.9\text{ nm}$; E, vitamin B₁₂, molecular mass 1.35 kDa), respectively. The inset shows an SDS–PAGE analysis of the elution peak. PaaA2_{13–63} and ParE2 are indicated by blue and red arrows, respectively. Lane M, Prestained Protein Molecular Weight Marker (Fermentas); lanes 1–3, fractions of the ParE2–PaaA2_{13–63} complex elution peak. (b) DLS. The black dots and grey line represent the experimental data and the fit, respectively. The residuals are shown below. The percentage of mass as a function of the R_h distribution is given in the inset. (c) The final scattering curve of the ParE2–PaaA2_{13–63} complex. The experimental data are shown in black, while the error margins are shown in grey. The inset shows the Guinier region of the scattering curve (experimental data in black and the Guinier fit in grey). (d) The normalized Kratky plot of the recorded data set indicates the presence of flexibility in the scattering particle. (e) An analysis of the $p(r)$ function demonstrates that the scattering particle has a maximum intra-particle distance of around 75 Å.

Table 2

Matthews analysis.

The analysis was conducted using the molecular mass of a ParE2–PaaA2 heterodimer (theoretical molecular mass 20.184 kDa) or a ParE2–PaaA2_{13–63} heterodimer (theoretical molecular mass 18.924 kDa). In both cases a 1:1 stoichiometry was assumed. AU and V_M are the asymmetric unit and the Matthews coefficient, respectively.

No. of heterodimers in the AU	V_M (Å ³ Da ^{−1})	Solvent content (%)	Probability
ParE2–PaaA2			
6	3.49	64.76	0.04
7	2.99	58.89	0.11
8	2.62	53.01	0.23
9	2.33	47.14	0.31
10	2.09	41.27	0.22
ParE2–PaaA2 _{13–63}			
1	5.93	79.28	0.00
2	2.97	58.57	0.49
3	1.98	37.85	0.50
4	1.48	17.14	0.00

initial screen had been set up, a single large crystal of up to 300 µm in length grew in 100 mM sodium acetate pH 5.0, 1 M ammonium sulfate (Molecular Dimensions Proplex 2 condition No. 20). The single ParE2–PaaA2_{13–63} crystal was cryo-quenched in liquid nitrogen with the addition of 15% (v/v) glycerol to the mother liquor as a cryoprotectant. The crystal diffracted to a resolution of ~2.7 Å. The diffraction pattern displays ice rings at 3.65 Å and reveals the presence of multiple lattices (Fig. 4b). Nonetheless, the most prominent lattice could be successfully indexed and integrated. The crystal belonged to space group $P6_122$ (or $P6_522$), with unit-cell parameters $a = b = 91.6$, $c = 185.6$ Å (data-collection statistics are given in Table 1 and the Matthews analysis in Table 2).

Structure determination of the ParE2–PaaA2 toxin–antitoxin complex *via* single-wavelength anomalous diffraction (SAD) appears encouraging. The $R_{\text{anom}}/R_{\text{p.i.m.}}$ ratio exceeds 1.5 ($R_{\text{anom}}/R_{\text{p.i.m.}} = 1.73$), which indicates that structure determination is likely to succeed (Weiss, 2001). In turn, structure determination of the ParE2–PaaA2 toxin–antitoxin complex in the $P4_12_12$ (or $P4_32_12$) crystal form will lead towards obtaining the crystal structures of the ParE2–PaaA2 complex in the $P3_12_1$ (or $P3_22_1$) crystal form and the ParE2–PaaA2_{13–63} heterodimer *via* molecular replacement. Combined with solution studies on the full-length ParE2–PaaA2 toxin–antitoxin complex (Sterckx *et al.*, 2012), this will finally shed light on the exact stoichiometry and architecture of this unusually large toxin–antitoxin oligomer, the biological function of which still remains elusive. Interestingly, our solution studies on the ParE2–PaaA2_{13–63} complex suggest that the first 12 N-terminal residues of PaaA2 play an important role in the assembly of the ParE2–PaaA2 oligomer. Based on the differences between the constructs used to produce the ParE2–PaaA2 and ParE2–PaaA2_{13–63} complexes, it seems that the displacement of the His tag on the toxin from the C-terminus to the N-terminus and/or the removal of the first 12 N-terminal residues from the antitoxin prevents the formation of a higher-order ParE2–PaaA2 oligomer. It is expected that a comparison between the crystal structures of the ParE2–PaaA2 and ParE2–PaaA2_{13–63} complexes will yield insights into the molecular driving forces behind the assembly (and disassembly) of the large ParE2–PaaA2 toxin–antitoxin oligomer.

This work was supported by FWO and by grants from OZR-VUB, the Hercules Foundation and VIB. YGJS received a predoctoral fellowship from FWO-Vlaanderen. LVM thanks the Fonds de la Recherche Scientifique (FRSM 3.4621.12) and the Interuniversity Attraction Poles Programme initiated by the Belgian Science Policy Office (MICRODEV) for financial support. The authors thank

Andrew Thompson and Beatriz Guimaraes for outstanding beamline support.

References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Bernard, P. & Couturier, M. (1992). *J. Mol. Biol.* **226**, 735–745.
- Blower, T. R., Salmond, G. P. C. & Luisi, B. F. (2011). *Curr. Opin. Struct. Biol.* **21**, 109–118.
- Blower, T. R., Short, F. L., Rao, F., Mizuguchi, K., Pei, X. Y., Fineran, P. C., Luisi, B. F. & Salmond, G. P. C. (2012). *Nucleic Acids Res.* **40**, 6158–6173.
- Brown, B. L., Grigoriu, S., Kim, Y., Arruda, J. M., Davenport, A., Wood, T. K., Peti, W. & Page, R. (2009). *PLoS Pathog.* **5**, e1000706.
- Castro-Roa, D., Garcia-Pino, A., De Gieter, S., van Nuland, N. A. J., Loris, R. & Zenkin, N. (2013). *Nature Chem. Biol.* **9**, 811–817.
- Christensen, S. K., Pedersen, K., Hansen, F. G. & Gerdes, K. (2003). *J. Mol. Biol.* **332**, 809–819.
- Christensen-Dalsgaard, M. & Gerdes, K. (2006). *Mol. Microbiol.* **62**, 397–411.
- Christensen-Dalsgaard, M., Jørgensen, M. G. & Gerdes, K. (2010). *Mol. Microbiol.* **75**, 333–348.
- Cooper, C. R., Daugherty, A. J., Tachdjian, S., Blum, P. H. & Kelly, R. M. (2009). *Biochem. Soc. Trans.* **37**, 123–126.
- Dao-Thi, M.-H., Van Melderden, L., De Genst, E., Afif, H., Buts, L., Wyns, L. & Loris, R. (2005). *J. Mol. Biol.* **348**, 1091–1102.
- David, G. & Pérez, J. (2009). *J. Appl. Cryst.* **42**, 892–900.
- De Jonge, N., Garcia-Pino, A., Buts, L., Haesaerts, S., Charlier, D., Zangger, K., Wyns, L., De Greve, H. & Loris, R. (2009). *Mol. Cell.* **35**, 154–163.
- Diederichs, K. & Karplus, P. A. (1997). *Nature Struct. Biol.* **4**, 269–275.
- Fischer, H., de Oliveira Neto, M., Napolitano, H. B., Polikarpov, I. & Craievich, A. F. (2010). *J. Appl. Cryst.* **43**, 101–109.
- Florek, P., Levnikov, V. M., Blagova, E., Lebedev, A. A., Škrabana, R., Resetárova, S., Pavelciová, P., Barak, I. & Wilkinson, A. J. (2011). *J. Biol. Chem.* **286**, 6808–6819.
- Garcia-Pino, A., Balasubramanian, S., Wyns, L., Gazit, E., De Greve, H., Magnuson, R. D., Charlier, D., van Nuland, N. A. J. & Loris, R. (2010). *Cell*, **142**, 101–111.
- Garcia-Pino, A., Christensen-Dalsgaard, M., Wyns, L., Yarmolinsky, M., Magnuson, R. D., Gerdes, K. & Loris, R. (2008). *J. Biol. Chem.* **283**, 30821–30827.
- Gerdes, K., Christensen, S. K. & Løbner-Olesen, A. (2005). *Nature Rev. Microbiol.* **3**, 371–382.
- Germain, E., Castro-Roa, D., Zenkin, N. & Gerdes, K. (2013). *Mol. Cell.* **52**, 248–254.
- Hallez, R., Geeraerts, D., Sterckx, Y., Mine, N., Loris, R. & Van Melderden, L. (2010). *Mol. Microbiol.* **76**, 719–732.
- Hanahan, D., Jessee, J. & Bloom, F. R. (1991). *Methods Enzymol.* **204**, 63–113.
- Hazan, R., Sat, B. & Engelberg-Kulka, H. (2004). *J. Bacteriol.* **186**, 3663–3669.
- Jiang, Y., Pogliano, J., Helinski, D. R. & Konieczny, I. (2002). *Mol. Microbiol.* **44**, 971–979.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Kamada, K. & Hanaoka, F. (2005). *Mol. Cell.* **19**, 497–509.
- Kolodkin-Gal, I., Hazan, R., Gaathon, A., Carmeli, S. & Engelberg-Kulka, H. (2007). *Science*, **318**, 652–655.
- Leplae, R., Geeraerts, D., Hallez, R., Guglielmini, J., Drèze, P. & Van Melderden, L. (2011). *Nucleic Acids Res.* **39**, 5513–5525.
- Loris, R. & Garcia-Pino, A. (2014). *Chem. Rev.* **114**, 6933–6947.
- Madl, T., Van Melderden, L., Mine, N., Respondek, M., Oberer, M., Keller, W., Khatai, L. & Zangger, K. (2006). *J. Mol. Biol.* **364**, 170–185.
- Maisonnette, E., Shakespeare, L. J., Jørgensen, M. G. & Gerdes, K. (2011). *Proc. Natl Acad. Sci. USA*, **108**, 13206–13211.
- Meinhart, A., Alonso, J. C., Sträter, N. & Saenger, W. (2003). *Proc. Natl Acad. Sci. USA*, **100**, 1661–1666.
- Moritz, E. M. & Hergenrother, P. J. (2007). *Proc. Natl Acad. Sci. USA*, **104**, 311–316.
- Mutschler, H., Gebhardt, M., Shoeman, R. L. & Meinhart, A. (2011). *PLoS Biol.* **9**, e1001033.
- Neubauer, C., Gao, Y.-G., Andersen, K. R., Dunham, C. M., Kelley, A. C., Hentschel, J., Gerdes, K., Ramakrishnan, V. & Brodersen, D. E. (2009). *Cell*, **139**, 1084–1095.
- Oberer, M., Zangger, K., Gruber, K. & Keller, W. (2007). *Protein Sci.* **16**, 1676–1688.
- Pedersen, K., Christensen, S. K. & Gerdes, K. (2002). *Mol. Microbiol.* **45**, 501–510.
- Pedersen, K., Zavialov, A. V., Pavlov, M. Y., Elf, J., Gerdes, K. & Ehrenberg, M. (2003). *Cell*, **112**, 131–140.

- Petoukhov, M. V., Franke, D., Shkumatov, A. V., Tria, G., Kikhney, A. G., Gajda, M., Gorba, C., Mertens, H. D. T., Konarev, P. V. & Svergun, D. I. (2012). *J. Appl. Cryst.* **45**, 342–350.
- Rambo, R. P. & Tainer, J. A. (2013). *Nature (London)*, **496**, 477–481.
- Ren, D., Walker, A. N. & Daines, D. A. (2012). *BMC Microbiol.* **12**, 263.
- Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W. & Mougous, J. D. (2011). *Nature (London)*, **475**, 343–347.
- Sat, B., Reches, M. & Engelberg-Kulka, H. (2003). *J. Bacteriol.* **185**, 1803–1807.
- Schifano, J. M., Edifor, R., Sharp, J. D., Ouyang, M., Konkimalla, A., Husson, R. N. & Woychik, N. A. (2013). *Proc. Natl Acad. Sci. USA*, **110**, 8501–8506.
- Shah, D., Zhang, Z., Khodursky, A., Kaldalu, N., Kurg, K. & Lewis, K. (2006). *BMC Microbiol.* **6**, 53.
- Smith, C. L., Ghosh, J., Elam, J. S., Pinkner, J. S., Hultgren, S. J., Caparon, M. G. & Ellenberger, T. (2011). *Structure*, **19**, 192–202.
- Sterckx, Y. G. J., Garcia-Pino, A., Haesaerts, S., Jové, T., Geerts, L., Sakellaris, V., Van Melderen, L. & Loris, R. (2012). *Acta Cryst.* **F68**, 724–729.
- Sterckx, Y. G. J., Volkov, A. N., Vranken, W. F., Kragelj, J., Jensen, M. R., Buts, L., Garcia-Pino, A., Jové, T., Van Melderen, L., Blackledge, M., van Nuland, N. A. J. & Loris, R. (2014). *Structure*, **22**, 854–865.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990). *Methods Enzymol.* **185**, 60–89.
- Szekeres, S., Dauti, M., Wilde, C., Mazel, D. & Rowe-Magnus, D. A. (2007). *Mol. Microbiol.* **63**, 1588–1605.
- Takagi, H., Kakuta, Y., Okada, T., Yao, M., Tanaka, I. & Kimura, M. (2005). *Nature Struct. Mol. Biol.* **12**, 327–331.
- Uversky, V. N. (1993). *Biochemistry*, **32**, 13288–13298.
- Vesper, O., Amitai, S., Belitsky, M., Byrgazov, K., Kaberdina, A. C., Engelberg-Kulka, H. & Moll, I. (2011). *Cell*, **147**, 147–157.
- Weiss, M. S. (2001). *J. Appl. Cryst.* **34**, 130–135.
- Weiss, M. S. & Hilgenfeld, R. (1997). *J. Appl. Cryst.* **30**, 203–205.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Winther, K. S. & Gerdes, K. (2011). *Proc. Natl Acad. Sci. USA*, **108**, 7403–7407.
- Yuan, J., Sterckx, Y., Mitchenall, L. A., Maxwell, A., Loris, R. & Waldor, M. K. (2010). *J. Biol. Chem.* **285**, 40397–40408.
- Zhang, Y. & Inouye, M. (2011). *Mol. Microbiol.* **79**, 1418–1429.
- Zhang, Y., Zhang, J., Hoeflich, K. P., Ikura, M., Qing, G. & Inouye, M. (2003). *Mol. Cell*, **12**, 913–923.
- Zhang, J., Zhang, Y. & Inouye, M. (2003). *J. Biol. Chem.* **278**, 32300–32306.
- Zhao, L. & Zhang, J. (2008). *FEBS Lett.* **582**, 710–714.
- Zwart, P., Grosse-Kunstleve, R. & Adams, P. (2005). *CCP4 Newsl. Protein Crystallogr.* **43**, contribution 7.