Production, biophysical characterization and crystallization of *Pseudomonas putida* GraA and its complexes with GraT and the *graTA* operator

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The *graTA* operon from *Pseudomonas putida* encodes a toxin–antitoxin module with an unusually moderate toxin. Here, the production, SAXS analysis and crystallization of the antitoxin GraA, the GraTA complex and the complex of GraA with a 33 bp operator fragment are reported. GraA forms a homodimer in solution and crystallizes in space group $P2_1$, with unit-cell parameters $a = 66.9$, $b = 48.9$, $c = 62.7$ Å, $\beta = 92.6^\circ$. The crystals are likely to contain two GraA dimers in the asymmetric unit and diffract to 1.9 Å resolution. The GraTA complex forms a heterotetramer in solution. Crystals of the GraTA complex diffracted to 2.2 Å resolution and are most likely to contain a single heterotetrameric GraTA complex in the asymmetric unit. They belong to space group $P4_1$ or $P4_3$, with unit-cell parameters $a = b = 56.0$, $c = 128.2$ Å. The GraA–operator complex consists of a 33 bp operator region that binds two GraA dimers. It crystallizes in space group $P3_1$ or $P3_2$, with unit-cell parameters $a = b = 105.6$, $c = 149.9$ Å. These crystals diffract to 3.8 Å resolution.

1. Introduction

Toxin–antitoxin (TA) modules are small operons that are involved in the response of bacteria and archaea to stress and in the establishment of the persister phenotype. They were initially discovered in the 1980s as isolated elements that are present on certain low-copy-number plasmids, where they contribute to the stabilization of these plasmids (Ogura & Hiraga, 1983; Gerdes *et al.*, 1986). Subsequently, it became apparent that besides being present on mobile DNA, TA modules are also widespread in the genomes of bacteria and archaea (Pandey & Gerdes, 2005; Leplae *et al.*, 2011), which initiated a strong debate about their true function in microbial biology. Possible roles attributed to TA modules include junk DNA and selfish alleles, the stabilization of genomic segments, protection against plasmids and phages, mediators of stress response including altruistic suicide modules, and persister cell formation (Magnuson, 2007).

Six different classes of TA modules have been described based on the nature of the toxin and antitoxin (for a review, see Kędzierska & Hayes, 2016). The best-studied class is formed by type II TA modules, where both the toxin and antitoxin are proteins and where the antitoxin neutralizes the toxin through direct interaction (for reviews, see Gerdes *et al.*, 2005; Yamaguchi *et al.*, 2011; Loris & Garcia-Pino, 2014; Page & Peti, 2016). The antitoxin is often a two-domain protein consisting of a folded DNA-binding/dimerization domain linked to a toxin-binding domain (for a review, see Loris &

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Garcia-Pino, 2014). However, in some cases the DNA-binding domain is absent or encoded as a separate gene (Zielenkiewicz & Ceglowski, 2005; Hallez *et al.*, 2010). The toxin-binding domain is usually intrinsically disordered, but some exceptions are again found (Brown *et al.*, 2009; Schureck *et al.*, 2014).

A large variety of toxins have been identified that target different cellular processes. Most toxins, nevertheless, seem to be RNases. The RelE superfamily, which also includes HigB, YoeB, MqsR and YafQ, requires translating ribosomes to cut the mRNA (Pedersen *et al.*, 2003; Christensen-Dalsgaard & Gerdes, 2008; Prysak *et al.*, 2009; Hurley & Woychik, 2009; Yamaguchi *et al.*, 2009). MazF toxins are ribonucleases that also target mRNA, but their activity is ribosome-independent (Zhang *et al.*, 2003; Christensen *et al.*, 2003). The ParE and CcdB toxins are gyrase poisons, but are structurally related to RelE and MazF, respectively (Jiang *et al.*, 2002; Yuan *et al.*, 2010; Bernard & Couturier, 1992; Dao-Thi *et al.*, 2005). Other toxins are kinases, AMPylases or acetyltransferases: Doc phosphorylates elongation factor Tu, while HipA phosphorylates glutamyl-tRNA synthetase (Castro-Roa *et al.*, 2013; Germain *et al.*, 2013), and ζ toxins such as PezT from *Streptococcus pneumoniae* and *S. pyogenes* phosphorylate the peptidoglycan precursor uridine diphosphate-*N*-acetylglucosamine (Mutschler *et al.*, 2011). Fic-like toxins from various bacteria AMPylate GyrB and ParE, the B subunits of DNA gyrase and topoisomerase IV (Harms *et al.*, 2015), while *Salmonella typhimurium* TacT acetylates elongation tRNA (Cheverton *et al.*, 2016) and *Escherichia coli* AtaT acetylates the initiation Met-tRNA^{fMet} (Jurénas *et al.*, 2017).

GraTA is a toxin-antitoxin module that was recently discovered in *Pseudomonas putida* (Tamman *et al.*, 2014). It exhibits the inverse gene order observed in the *higBA* family of modules. Among the structures present in the Protein Data Bank, the antitoxin GraA shows 29% sequence identity to the HigA antitoxin from *Proteus vulgaris* (PDB entry 4mcx; Schureck *et al.*, 2014) and 27% and 42% sequence identity to the transcription regulator YddM from *E. coli* (PDB entries 2ict and 2icp; Arbing *et al.*, 2010) and the lone HigA-like virulence-associated protein from *Coxiella burnetii* (PDB entry 3trb; Franklin *et al.*, 2015), respectively. The toxin GraT shows 29% sequence identity to HigB from *P. vulgaris* (PDB entry 4mcx; Schureck *et al.*, 2014). While GraT seems to interfere with ribosome biogenesis (Ainelo *et al.*, 2016), the exact mechanism of its toxicity remains unknown. The GraT toxin is distinct from other HigB homologues because of its unusually mild and temperature-dependent effects. Deletion of the *graA* gene affects the growth rate of *P. putida* mainly at lower temperatures and becomes lethal only when *graT* is effectively overexpressed (Tamman *et al.*, 2014). Degradation of GraA, the presumed trigger to activate GraT, is independent of Lon or Clp (Tamman *et al.*, 2015), the proteases that usually target type II antitoxins and that are thought to be central in the cascade that leads to the persister phenotype in *E. coli* (Maisonneuve *et al.*, 2013; Maisonneuve & Gerdes, 2014; Germain *et al.*, 2015).

In this paper, we report the crystallization of GraA, the GraTA complex and the complex of GraA with the 33 bp

Table 1
Macromolecule-production information.

GraTA complex	
Source organism	<i>P. putida</i> strain PaW85 (isogenic to strain KT2440)
DNA source	<i>P. putida</i> strain PaW85
Forward primer (T-Nhis)	CTCCATATGCATCACCACCACCATCATTGCGAA-GCTTTAGCTGT
Reverse primer (1585bam)	ATGGATCCGTTTTTCGATGTCAGT
Cloning vector	pET-11c
Expression vector	pET-11c
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	
GraT	MHHHHHHIRSFSCADTEALFTTGKTRRGSDIKSV-AERKLAMLDAAETELRDLRSPGNRLESLSGNNR-ADQHSIRVNDQWRLCFTWTEHGPVNVEIVDYHMLKNGMRPIHPGEILREEFKEMGFSAALARAL-GVATPTVNNILRERGVSADMLRLSICLDTT-PEFWLNLQTAFLDLRTAEQHGDEIGSVQRLVA
GraA	
GraA	
Source organism	<i>P. putida</i> strain PaW85
DNA source	<i>P. putida</i> strain PaW85
Forward primer (A-Nhis)	AATCATATGCATCACCACCACCATCACCCTCAAGA-ACGGTATCGCTCC
Reverse primer (1585bam)	ATGGATCCGTTTTTCGATGTCAGT
Cloning vector	pET-11c
Expression vector	pET-11c
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MHHHHHHKNGMRPIHPGEILREEFKEMGFSAALARALGVATPTVNNILRERGVSADMLRLSICLDTTPEFWLNLQTAFLDLRTAEQHGDEIGSVQRLVA

operator. The resulting structures are expected to help us in our investigations of the role, mode of function and regulation of the *P. putida graTA* module and to better understand the evolutionary relationships within the RelE superfamily.

2. Materials and methods

2.1. Purification of the GraTA complex

The GraTA complex, consisting of N-terminally tagged GraT and untagged GraA, was expressed from plasmid pET-hisTA (Tamman *et al.*, 2014). *E. coli* BL21(DE3) cultures carrying pET-hisTA (Table 1) were grown in LB medium at 30°C to an OD₅₈₀ of ~0.3. The temperature was then shifted to 25°C and protein expression was induced with 0.5 mM IPTG at an OD of ~0.6. After 4–5 h of induction, the cells were pelleted and sonicated in buffer A (50 mM Tris pH 8.0, 0.25 M NaCl, 2 mM β -mercaptoethanol). Cellular debris was removed by centrifugation for 20 min at 16 000g at 4°C. The supernatant was filtered through a 0.22 μ m filter before loading it onto a 1 ml HisTrap HP column (GE Healthcare Life Sciences) equilibrated with buffer A.

The column was washed with buffer A until the absorbance of the flowthrough at 280 nm approached the baseline. Proteins were eluted from the column using a five-step gradient to buffer B (50 mM Tris pH 8.0, 250 mM NaCl, 2 mM β -mercaptoethanol, 500 mM imidazole). The steps consisted of five column volumes of 2, 5, 10, 50 and 100% buffer B. The protein-containing fractions were immediately loaded onto a Bio-Rad SEC 70 gel-filtration column equilibrated with buffer A. The pure protein fractions were checked by SDS-PAGE,

Table 2

DNA duplexes used for crystallization. The 2×6 bp inverted repeat is underlined.

Duplex 1 (blunt)	5' AATCGAAATTAACGAATAACGTTAAGCATTTCAGCTCATG 3'
	3' TTAGCTTTAATTGCTTATTGCAATTCGTAAGTCGAGTAC 5'
Duplex 2 (blunt)	5' AAATTAACGAATAACGTTAAGCATTTCAGCTCAT 3'
	3' TTTAATTGCTTATTGCAATTCGTAAGTCGAGTA 5'
Duplex 3 (1 bp 5' overhang)	5' TAAATTAACGAATAACGTTAAGCATTTCAGCT 3'
	3' -TTAATTGCTTATTGCAATTCGTAAGTCGAA 5'

flash-frozen in liquid nitrogen and stored at -80°C until further use.

2.2. Purification of GraA and preparation of GraA₂–DNA complexes

N-terminally histidine-tagged GraA was expressed from the plasmid pET-hisA (see Table 1 for details; Tamman *et al.*, 2014). Otherwise, purification of GraA was performed exactly as described above for the GraTA complex.

Three different DNA duplexes were used in screening crystallization conditions for the GraA–operator complex (Table 2). To prepare these duplexes, single-stranded oligonucleotides were purchased from Sigma–Aldrich with additional reverse-phase cartridge purification. The oligonucleotides were solubilized in water at $\sim 200\ \mu\text{M}$. Each pair of complementary strands was mixed in a 1:1 ratio to obtain a final concentration of $\sim 100\ \mu\text{M}$ for the double-stranded DNA. The mixtures were heated to 80°C for 20 min and slowly cooled to room temperature. The formation of all double-stranded DNAs was checked by gel filtration. The GraA₂ dimer was mixed with each of the three different operator variants at a GraA₂:duplex DNA molar ratio of 2.2:1. The excess protein was removed by gel filtration using a Bio-Rad SEC 70 column equilibrated and run in 50 mM Tris pH 8.0, 250 mM NaCl, 2 mM β -mercaptoethanol.

2.3. Crystallization

GraA and GraTA were stored at -80°C in 50 mM Tris pH 8.0, 0.25 M NaCl, 2 mM β -mercaptoethanol. After thawing in a thermoblock at 37°C , the protein samples were concentrated to $10\ \text{mg ml}^{-1}$ by centrifugation using Amicon 10 kDa centrifugal filters. Concentrated protein–DNA solutions were immediately subjected to crystallization trials after preparation. Crystallization conditions were screened at 20°C by the sitting-drop vapour-diffusion method using three-well Intelli-Plates and a Phoenix liquid-handling system (Art Robbins). Sitting drops consisted of 0.1 μl protein–DNA complex solution and 0.1 μl reservoir solution and were equilibrated against 55 μl reservoir solution. Crystallization conditions were tested with several commercially available screens: Crystal Screen, Crystal Screen 2 and PEGRx (Hampton Research), ProPLEX and JCSG (Molecular Dimensions), and JBScreen Classic 1–4 (Jena Biosciences).

2.4. Mass-spectrometric analysis

Protein samples were reduced by the addition of 5 mM DTT, followed by incubation for 1 h at 56°C . Subsequently, a

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

	GraA	GraA–DNA	GraTA
Diffraction source	SOLEIL	SOLEIL	SOLEIL
Beamline	PROXIMA-1	PROXIMA-2A	PROXIMA-1
Wavelength ($\text{\AA}$)	0.978	0.979	0.978
Temperature (K)	100	100	100
Detector	PILATUS 6M	EIGER	PILATUS 6M
Crystal-to-detector distance (mm)	345	800	426
Rotation range per image ($^{\circ}$)	0.2	0.1	0.2
Total rotation range ($^{\circ}$)	180	180	220
Space group	$P2_1$	$P3_1$ or $P3_2$	$P4_1$ or $P4_3$
a, b, c ($\text{\AA}$)	66.9, 48.9, 62.7	105.6, 105.6, 149.9	56.0, 56.0, 128.2
α, β, γ ($^{\circ}$)	90.0, 92.6, 90.0	90.0, 90.0, 120.0	90.0, 90.0, 90.0
Mosaicity ($^{\circ}$)	0.17	0.16	0.09
Resolution range ($\text{\AA}$)	46.8–1.96 (2.03–1.96)	43.85–3.79 (3.92–3.79)	42.18–2.49 (2.58–2.49)
Total No. of reflections	287046 (42955)	96634 (9078)	134815 (12942)
No. of unique reflections	57483 (8695)	18427 (1797)	13706 (1354)
Completeness (%)	98.4 (93.3)	99.54 (95.69)	99.87 (98.90)
Multiplicity	5.0 (4.9)	5.2 (5.1)	9.8 (9.6)
$\langle I/\sigma(I) \rangle$	12.25 (2.08)	8.84 (0.91)	16.82 (2.05)
R_{merge}	0.088 (0.630)	0.130 (1.462)	0.110 (2.094)
R_{meas}	0.099 (0.704)	0.137 (1.492)	0.116 (2.210)
$CC_{1/2}$	1.00 (0.84)	0.99 (0.39)	1.00 (0.61)
CC^*	1.00 (0.92)	1.00 (0.74)	1.00 (0.82)
Overall B factor from Wilson plot ($\text{\AA}^2$)	34.0	156.4	59.5

final concentration of 25 mM iodoacetamide was added and the samples were incubated for a further 30 min at room temperature. The proteins were then digested with MS-grade trypsin (Thermo Fisher Scientific) at a final concentration of $0.04\ \text{mg ml}^{-1}$ for 18 h at 37°C . After digestion, the samples were analyzed on a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) in a shotgun analysis-type experiment following reverse-phase liquid chromatography. Native mass spectra were recorded on a travelling wave Q-TOF instrument (Synapt G2, Waters, Manchester, England) tuned for the transmission of large, native protein assemblies. The voltages used were as follows: sampling cone, 50 V; trap collision cell, 75 V; trap DC bias, 45 V.

2.5. X-ray data collection and analysis

Crystals were flash-cooled in liquid nitrogen after transferring them to a suitable cryoprotectant solution (Table 3). All data were measured at the SOLEIL synchrotron facility, Gif-sur-Yvette, France. For the GraA and GraTA crystals the diffraction data were measured on the PROXIMA-1 beamline using a wavelength of $0.978\ \text{\AA}$ and were recorded on a PILATUS 6M detector. Data for the GraA–operator complex were collected on beamline PROXIMA-2A using an EIGER detector and a wavelength of $0.979\ \text{\AA}$. All data were indexed, integrated and scaled with *XDS* (Kabsch, 2010) via the *xdsmc* interface. Data quality and twinning were verified with *phenix.xtriage* (Zwart *et al.*, 2005) and *POINTLESS* (Evans, 2006).

2.6. Analytical size-exclusion chromatography

Analytical size-exclusion chromatography of GraA₂, GraT₂A₂ and GraA₂-DNA was carried out on a Bio-Rad Enrich SEC 70 column equilibrated with 50 mM Tris pH 8.0, 250 mM NaCl, 2 mM β -mercaptoethanol. The injections were of 0.5 ml and the flow rate was 1 ml min⁻¹. To calibrate the column, we used BSA (69 kDa; Sigma-Aldrich, catalogue No. A2153) and a mixture of chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa) (Bio-Rad, catalogue No. 151-1901). Molecular weights were estimated according to Whitaker (1963).

2.7. Small-angle X-ray scattering (SAXS)

SAXS data for the GraTA complex (14 mg ml⁻¹) as well as the GraA-DNA complex (duplex 2 in Table 2; 3.6 mg ml⁻¹ protein and 50 μ M DNA) were collected on the SWING beamline at the SOLEIL synchrotron, while SAXS data for GraA (7 mg ml⁻¹) were collected on beamline BM29 at the ESRF. These beamlines have a size-exclusion chromatography (SEC) system coupled to the measuring capillary. SEC removes possible aggregates, rendering a very homogeneous sample that is then directly exposed to X-rays for data collection. For this experiment, we used a Shodex KW404-4F column that was run at 0.2 ml min⁻¹. A solution consisting of 50 mM Tris pH 8.0, 250 mM NaCl, 2 mM TCEP was used as running buffer. The scattering curves, covering a concentration range around the peak, are averaged to obtain the final scattering curve. R_g values were obtained from Guinier

approximation and the values for I_0 were obtained from extrapolation of the Guinier region to $q = 0$ as implemented in the ATLAS suite (Petoukhov *et al.*, 2012).

3. Results and discussion

3.1. Production of GraA and GraTA

GraA and the GraA-GraT complex (GraTA) were both expressed in *E. coli* BL21 (DE3) cells using pET-11c expression vector. Both N-terminally His-tagged GraA and the GraTA complex (with a His tag at the N-terminus of GraT) could be purified to homogeneity by Ni-NTA affinity-purification chromatography followed by gel filtration. From a single litre of culture, we were able to obtain 10 mg of pure GraA or 8 mg of the GraTA complex. The preparations were >99% pure as judged by SDS-PAGE (Fig. 1), with GraA running at an apparent molecular weight of approximately 11–12 kDa, which is in good agreement with the theoretical value of 11.7 kDa predicted from its amino-acid sequence. In the GraTA complex GraA runs slightly faster, in agreement with the lack of a His tag. 6His-GraT runs at an apparent molecular weight of approximately 13 kDa, which is somewhat higher than the 11.2 kDa calculated from its amino-acid sequence. The identities of both proteins were further confirmed by LC-MS/MS mass spectrometry from sliced-out gel bands subjected to trypsin digestion. The presence of a His tag was confirmed using Western blotting.

3.2. Characterization and crystallization of GraA

Prior to crystallization, the antitoxin GraA was characterized using analytical size-exclusion chromatography. GraA elutes from a Bio-Rad Enrich SEC 70 column at a volume of 11.2 ml, which corresponds to an estimated molecular weight of 26.4 kDa (Fig. 2). This suggests that GraA forms dimers (GraA₂), assuming that the particle adopts a globular shape

Figure 1
Purification of GraA and GraTA. SDS-PAGE of purified GraA (*a*) and the GraTA complex (*b*). Each protein migrates as a single band at its expected molecular weight. The Thermo Fisher PageRuler 10–180 kDa molecular-weight marker is shown as a reference (lane *M*).

Figure 2
Analytical size-exclusion chromatography. The SEC profiles of GraA (red), GraTA (black) and the GraA-operator complex (blue) obtained on a Bio-Rad Enrich SEC 70 column are shown, together with the elution positions of the molecular-weight standards (inverted triangles: BSA, 69 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 17 kDa; vitamin B₁₂, 1.35 kDa). The elution volumes of the peaks are indicated.

(the theoretical molecular weight of a GraA₂ dimer based on its amino-acid sequence is 23.4 Da). Since many antitoxins contain significant stretches of intrinsic disorder, we further analyzed GraA using HPLC–SAXS (Fig. 3*a*). The measured scattering curve is rather noisy and is only reliable up to a q

value of $0.15 \text{ \AA}^{-1}$, which is only sufficient for a Guinier analysis. The Guinier plot, although noisy, is linear and the calculated radius of gyration (R_g) of $20.0 \text{ \AA}$ is independent of concentration, indicating the absence of aggregation in the samples. The molecular weight estimated from extrapolation

Figure 3
Small-angle X-ray scattering. Background-subtracted scattering curves (left) and Guinier plots (right) for (a) GraA, (b) the GraTA complex and (c) the GraA-operator complex.

to I_0 is 27 kDa. This again is compatible with GraA forming a globular dimer in solution.

Initial screening for crystallization conditions for GraA led to the identification of six hits in related conditions from the ProPlex, Crystal Screen and Crystal Screen 2, and JBScreen Classic screens (Supplementary Table S1). The morphology of these initial crystals (small needles) was identical in all six conditions. These conditions were refined and crystals suitable for X-ray diffraction were obtained in 0.1 M KCl, 0.1 M

HEPES pH 7.5, 15% (w/v) PEG 6000. These crystals diffract to around 1.9 Å resolution (Fig. 4a). Data-collection statistics are given in Table 3. Data collected on beamline PROXIMA-1 of the SOLEIL synchrotron indexed as primitive monoclinic, with unit-cell parameters $a = 66.9$, $b = 48.9$, $c = 62.7$ Å, $\beta = 92.6^\circ$. Systematic absences are indicative of a 2_1 screw axis, indicating space group $P2_1$. Matthews calculations (Matthews, 1968; Kantardjieff & Rupp, 2003) show that the most probable content of the asymmetric unit would be two GraA dimers ($V_M = 2.17$ Å³ Da⁻¹, 43% solvent content, probability 0.97).

The GraA crystals are very small and we failed to wash these crystals in such a way that we can be certain that the remaining sample would not contain significant amounts of protein from the surrounding mother liquor. MS/MS analysis of tryptic digests of our GraA preparations indicate the presence of some contaminants, but with a very low abundance compared with GraA, probably at a concentration that is too low for them to be able to produce macroscopic crystals. Indeed, the isolated protein appears >99% pure as assessed by Coomassie-stained SDS-PAGE, which does not show the presence of additional bands corresponding to contaminating proteins. In order to further ascertain that the correct protein was crystallized, we submitted the data to the *ContaMiner* server (Hungler *et al.*, 2016). This confirmed that the diffraction data sets do not correspond to any of the most common protein contaminants from *E. coli*.

3.3. Characterization and crystallization of the GraTA complex

The GraTA complex eluted from the Bio-Rad Enrich SEC 70 column at a volume of 10.5 ml, which corresponds to an

Figure 4

Diffraction patterns of the crystals used for data collection for (a) GraA, (b) GraTA and (c) the GraA–operator complex. For clarity, only the upper left quarter of the detector is shown in each case.

estimated molecular weight of 38 kDa (Fig. 2). Therefore, the most likely stoichiometry of this complex is GraA₂–GraT₂, which is similar to the stoichiometry observed for the HigBA complexes from *P. vulgaris* and *E. coli* (Schureck *et al.*, 2014; Yang *et al.*, 2016) and other toxin–antitoxin complexes with toxins belonging to the RelE superfamily (Bøggild *et al.*, 2012; Brown *et al.*, 2009; Liang *et al.*, 2014; Ruangprasert *et al.*, 2014). This result was confirmed by HPLC–SAXS (Fig. 3b). In this case, the scattering curve has a very good signal-to-noise ratio to a q value of $0.65 \text{ \AA}^{-1}$. The R_g is calculated to be 23 Å, and the molecular weight estimated from extrapolation to I_0 is 41 kDa, in agreement with the analytical SEC results.

Crystallization conditions for the GraTA complex were screened at 10 mg ml^{-1} and 20°C . Initial hits were obtained in five distinct conditions (Supplementary Table S1). Of these, the crystals in a single condition [$0.8 \text{ M Li}_2\text{SO}_4$, 0.1 M sodium acetate trihydrate pH 4.0, 4% (v/v) PEG 200 at 20°C] were of sufficient size and quality to be used directly without further optimization. These crystals belong to space group $P4_1$ or $P4_3$, with unit-cell parameters $a = b = 56.03$, $c = 128.19 \text{ \AA}$. The best crystal diffracted to $2.2 \text{ \AA}$ resolution (Fig. 4b) and a full data set was collected on the PROXIMA-1 beamline at the SOLEIL synchrotron (Table 3). Matthews analysis indicates the presence of a single GraA₂–GraT₂ complex in the asymmetric unit of the crystal (V_M of $2.28 \text{ \AA}^3 \text{ Da}^{-1}$, 46% solvent content, probability 1.0). Again, the identity of the crystallized species was assessed as described for the GraA crystals, and both the purity of the protein sample and the results of querying the *ContaMiner* database, make it unlikely that the crystals arise from a contaminating protein.

3.4. Characterization and crystallization of the GraA–operator complex

GraA, but not the GraTA complex, represses the *graTA* operon and GraA protects a 33 bp operator region that contains a TAAACGAATAACGTTAA inverted repeat against DNase I cleavage (Tamman *et al.*, 2014). The complex of GraA with this 33 bp operator region (duplex 2 in Table 2) elutes from the Bio-Rad Enrich SEC 70 column at a volume of 9.3 ml, corresponding to a molecular weight of 71 kDa (Fig. 2). Knowing that the molecular mass of the DNA used is 20.4 kDa and that GraA forms a dimer of about 26 kDa, the stoichiometry of this complex is most likely to be GraA₂–DNA–GraA₂. This result was confirmed by HPLC–SAXS (Fig. 3c), which gives an R_g of 29 Å and an estimated molecular mass of 68.9 kDa. This is rather surprising given that the operator region contains only a single 2×6 bp inverted repeat, which is the most likely direct target of GraA.

We used three variants of the operator sequence to produce GraA–DNA complexes and screen for crystallization conditions (Table 2). The GraA–DNA complexes were prepared by adding a 2.2-fold molar excess of GraA dimers to the DNA duplex, followed by purification by gel filtration. Only the complex involving the sequence duplex 2 (see Table 2) yielded crystallization hits (Supplementary Table S1).

Most crystals of the GraA–DNA complex showed poor or no diffraction, with only few giving rise to diffraction spots exceeding $4 \text{ \AA}$ resolution. The best crystal diffracted to $3.8 \text{ \AA}$ resolution (Fig. 4c). This resolution estimate may be optimistic as we used less conservative criteria than for GraA and GraTA. Nevertheless, a $CC_{1/2}$ of 0.39 indicates the presence of significant information in the highest resolution shell even if the $I/\sigma(I)$ value is already slightly below 1 (Karplus & Diederichs, 2012; Diederichs & Karplus, 2013). The exact resolution to which the data are useful will have to be determined during refinement. Data-collection statistics are shown in Table 3. This crystal was grown in 0.2 M lithium sulfate, 0.1 M sodium acetate pH 4.5, 50% PEG 400. The crystal belongs to space group $P3_1$ or $P3_2$, with unit-cell parameters $a = b = 105.6$, $c = 149.9 \text{ \AA}$. Matthews calculations assuming a molecular species consisting of two GraA₂ dimers bound to a single DNA duplex indicates that it is most likely that there are three such complexes ($V_M = 2.33 \text{ \AA}^3 \text{ Da}^{-1}$, 47% solvent, probability 0.82) present in the asymmetric unit of the crystals.

To ascertain the presence of both GraA and DNA in the crystals, 15 crystals were washed in precipitant solution and then redissolved in 20 µl of water. Analysis of such redissolved crystals by native mass spectrometry indicated the presence of both GraA and the DNA fragment (Supplementary Fig. S1). In addition, 20 µl of the redissolved crystals was spotted onto a glass plate together with a DNA sample, a GraA sample and buffer as positive and negative controls, and 2 µl ethidium bromide was subsequently added, after which the drops were illuminated with UV light. While the GraA preparation did not enhance the UV fluorescence of ethidium bromide, the redissolved GraA–DNA crystals did, similar to the DNA control, indicating the presence of significant amounts of DNA in these crystals (Supplementary Fig. S1).

4. Conclusion

graTA is a toxin–antitoxin module from *P. putida* that has been shown to interfere with ribosome biogenesis (Ainelo *et al.*, 2016). While the sequence similarity of both the toxin and antitoxin to the components of *P. vulgaris* *higBA* suggests GraT to be a ribonuclease, its effective mode of action, regulation and the reason for its unusually mild effect on bacterial growth remain unknown. We have expressed, produced and crystallized GraA, the GraTA complex and a GraA–operator complex. The corresponding crystal structures are likely to enhance our understanding of the function and regulation of the *graTA* toxin–antitoxin module.

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