Crystallization of two operator complexes from the *Vibrio cholerae* HigBA2 toxin–antitoxin module

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The HigA2 antitoxin and the HigBA2 toxin–antitoxin complex from *Vibrio cholerae* were crystallized in complex with their operator box. Screening of 22 different DNA duplexes led to two crystal forms of HigA2 complexes and one crystal form of a HigBA2 complex. Crystals of HigA2 in complex with a 17 bp DNA duplex belong to space group $P3_221$, with unit-cell parameters $a = b = 94.0$, $c = 123.7$ Å, and diffract to 2.3 Å resolution. The second form corresponding to HigA2 in complex with a 19 bp duplex belong to space group $P4_32_12$ and only diffract to 3.45 Å resolution. Crystals of the HigBA2 toxin–antitoxin were obtained in complex with a 31 bp duplex and belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 113.6$, $c = 121.1$ Å. They diffract to 3.3 Å resolution.

1. Introduction

The adaptation of bacterial cells to environmental stress is a complex biochemical endeavour that requires the integration of different extracellular and intracellular signals. Subsequent changes in gene expression and activation of stress-response factors may lead to the establishment of the persister state: a metabolically calm, nongrowing state that allows antibiotic treatments to be survived (Lewis, 2010). One group of proteins that are up-regulated during stress response and that are involved in persistence are toxin–antitoxin (TA) modules (Christensen *et al.*, 2003; Cooper *et al.*, 2009; Christensen-Dalsgaard *et al.*, 2010; Maisonneuve & Gerdes, 2014). These small operons consist of two genes, one coding for a ‘toxin’ and the other for an ‘antitoxin’ protein (for reviews, see Gerdes *et al.*, 2005; Buts *et al.*, 2005; Yamaguchi *et al.*, 2011). In unstressed cells, the toxin and antitoxin form a noncovalent complex. A sufficiently low antitoxin:toxin ratio is maintained through a regulatory mechanism called conditional cooperativity (Overgaard *et al.*, 2008; De Jonge *et al.*, 2009; Garcia-Pino *et al.*, 2010; Afif *et al.*, 2001). In stressed cells, on the other hand, the activation of intracellular proteases leads to degradation of the antitoxin. The resulting activation of the toxin brings the cell to metabolic arrest (Hansen *et al.*, 2012; Christensen-Dalsgaard *et al.*, 2010).

Regulation by conditional cooperativity is linked to the regulation of protein activity *via* a very strong interaction between the toxin and antitoxin (Cataudella *et al.*, 2012; Gelens *et al.*, 2013) and most often depends on the intrinsically disordered nature of the antitoxin (reviewed in Loris & Garcia-Pino, 2014). Molecular mechanisms by which conditional cooperativity functions have been suggested for several

TA modules and involve the formation of different TA complexes depending on the toxin:antitoxin ratio (Garcia-Pino *et al.*, 2010; De Jonge *et al.*, 2009; Winther & Gerdes, 2012). These complexes differ in their affinities for the operator. Thus, the toxin functions either as a co-repressor or as an activator. Current models emphasize the importance of the cooperative interactions between TA complexes at the operator sites. In complexes with low T:A ratios, cooperative interactions increase the affinity for the operator. On the other hand, for complexes which form at high T:A ratios the affinity for the operator sequence is decreased owing to steric repulsion between the TA complexes at the different operator sites. The details of cooperativity are not well understood, partially owing to a lack of high-resolution structures of these complexes. To date, the only two TA modules for which toxin–antitoxin–operator structures are available are VapBC and HipBA, which are probably not regulated *via* conditional cooperativity (Schumacher *et al.*, 2009; Maté *et al.*, 2012; Mattison *et al.*, 2006; Wen *et al.*, 2014). In both modules the two binding sites on the operator are occupied by antitoxin dimers, while the antitoxin-bound toxins are positioned perpendicularly relative to the DNA molecule. This forms a circular repressor complex distinct from that predicted for other TA modules where conditional cooperativity has been well validated (such as RelBE, CcdAB and Phd–Doc). Structural information on toxin–antitoxin–operator complexes from other TA families would thus provide important clues about the regulation of TA modules as well as the regulation of small bacterial operons in general.

In this paper, we report the crystallization of two bacterial auto-regulators from the HigBA2 toxin–antitoxin module, the antitoxin HigA2 and the toxin–antitoxin complex HigBA2, in complex with their operator DNA. In the first step, we identified the oligonucleotide sequences that are compatible with crystal growth. In the second step, we used another set of oligonucleotides with slight variations to the original sequence to optimize the diffraction quality.

2. Materials and methods

2.1. Preparation of protein–DNA complexes

The antitoxin HigA2 and the complex between HigA2 and HigB2 (His-HigBA2 with His-tag N-terminal to HigB2) were prepared as described previously (Hadži *et al.*, 2013). Briefly, both His-HigB2 and HigA2 were coexpressed in *Escherichia coli* BL21 (DE3) cells harboring a plasmid pET15b (Novagen). After induction, the cell lysate was loaded onto an Ni–NTA column and nonspecifically bound proteins were eluted using a buffer with high ionic strength. At this step the whole complex was either eluted using an imidazole gradient or when separated proteins were needed a separation protocol was applied. In the latter case the complex was disrupted using a buffer supplemented with 6 M guanidinium hydrochloride, followed by on-column refolding using a buffer containing 5% glycerol. Finally, both the toxin and the antitoxin were eluted with an imidazole gradient as two separate peaks. A detailed description can be found in Hadži *et al.* (2013).

Table 1

Macromolecule-production information.

Source organism	<i>V. cholerae</i> strain N16961
DNA source	<i>V. cholerae</i> strain N16961
Forward primer†	CCCCCATATGAAAAGTGTATTGTGCAATCAAC
Reverse primer‡	CCCCCGGATCCGTTATAGCTCGGCTATGTGTG
Cloning vector	pOPTHM
Expression vector	pET-15b+
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced§	MGSSHHHHHHSSGLVPRGSHMKSVMFVESTIFEKY-RDEYLSDEEYRLFQAEMLNPKLGDVIGQTGG-LRKIRVASKGKGRGSRRIYYFLDEKRRFYLLTIYGNEMSDLNANQRKQLMAFMEAWRNEQS-MSNRDLFAELSSALVEAKHSEGLTLKTHHV-NDVGELNISPDEIVSIREQFNMSRGVFAILLH-TSSRTLLENWEQGRSVPNGQAVTLLKLVQRHPE-TLSHIAEL

† The NdeI cleavage site is underlined. ‡ The BamHI cleavage site is underlined. § The His tag and the thrombin cleavage site are underlined; the amino-acid sequence for the HigB2 toxin is written in regular font followed by the amino-acid sequence for the HigA2 antitoxin in italics. The last four amino-acid residues of HigB2 overlay with the first four residues from HigA2 at the DNA level. When expressed, the HigA2 antitoxin lacks the initiator methionine.

In addition to the full-length His-HigBA2 complex, a variant with the N-terminal affinity tag cleaved off was produced (called HigBA2ΔHis in the following). Cleavage was performed overnight at room temperature by mixing with one unit of thrombin (GE Healthcare) per milligram of the His-HigBA2 complex. The HigBA2ΔHis complex was further purified on a Superdex 75 HR 10/30 gel-filtration column (GE Healthcare) in 20 mM Tris pH 8, 15 mM magnesium chloride, 200 mM sodium chloride. The column was loaded with a 100 µl sample containing 0.5 mg of cleaved products and run at 0.5 ml min^{−1}.

DNA oligonucleotides were obtained from Sigma–Aldrich with additional reverse-phase cartridge purification. Oligonucleotides were dissolved to 2 mM in triple-distilled water and mixed stoichiometrically with the complementary strand. The mixture was heated to 95°C and left to slowly cool to room temperature while the duplexes annealed. Before crystallization, the protein stocks (in 20 mM Tris pH 8, 0.2 M sodium chloride and 15 mM magnesium chloride) were concentrated with 3 kDa molecular-weight cutoff centrifugal filters (Amicon Ultra UltraCel) to final concentrations of 10 mg ml^{−1} for His-HigBA2 and of 8 mg ml^{−1} for both HigBA2ΔHis and HigA2. Concentrated proteins were mixed with excess DNA duplexes in a 1:1.2 molar ratio. The final concentrations of the protein–DNA complexes were 160 µM for His-HigBA2, 120 µM for HigBA2ΔHis and 250 µM for HigA2, assuming that one molecule (a HigBA2 tetramer or a HigA2 dimer) interacts with the DNA duplex.

2.2. Crystallization

Concentrated protein–DNA solutions were immediately subjected to crystallization trials. 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.

Table 2

Oligonucleotides used for initial screening.

Palindromic repeats are underlined.

No.	Sequence	Length	Ends
1	GTACGCTTGGT <u>GCGTAC</u>	17	Blunt
2	CTGTACGCTTGGT <u>GCGTACAC</u>	21	Blunt
3	TTGTACGCTTGGT <u>GCGTACAC</u>	21	T/A
4	TCTGTACGCTTGGT <u>GCGTACAC</u>	22	T/A
5	ATCTGTACGCTTGGT <u>GCGTACACTT</u>	25	Blunt
6	ATCTGTACGCTTGGT <u>GCGTACACTTA</u>	26	Blunt
7	TATCTGTACGCTTGGT <u>GCGTACACTT</u>	26	T/A
8	GCCATCTGTACGCTTGGT <u>GCGTACACTTCCT</u>	31	Blunt
9	TGCCATCTGTACGCTTGGT <u>GCGTACACTTCC</u>	31	T/A

Crystallization conditions were tested with several commercially available screens: Natrix 1 and 2, PEGRx and Index from Hampton Research, ProPlex, Morpheus (Gorrec, 2009) and JSCG-*plus* from Molecular Dimensions and Protein–Nucleic Acid Complex screen from KeraFast (Pryor *et al.*, 2012).

2.3. X-ray data collection and analysis

All data were measured at the SOLEIL synchrotron facility, Gif-sur-Yvette, France. For HigBA2ΔHis–DNA crystals the diffraction data were measured on the PROXIMA2 beamline using a wavelength of 0.9801 Å and the ADSC detector. By rotating the crystal in 0.5° increments, 180 images were collected in total.

Diffraction data for both crystal forms of the HigA2–DNA complex were collected on the PROXIMA1 beamline. Data were collected using a wavelength of 0.9791 Å and were recorded on a PILATUS 6M detector. Crystals containing the antitoxin and a 17 bp duplex (space group $P3_121$) were rotated in 0.1° increments over 200°. For crystals containing the antitoxin and a 19 bp duplex (space group $P4_32_12$), 1000 images were collected by rotating the crystal in 0.2° increments. All data were indexed, integrated and scaled with *XDS* (Kabsch, 2010). Data quality was verified with *phenix.xtriage* (Zwart *et al.*, 2005).

2.4. Molecular replacement

Structures of HigA2 in complex with the operator were determined by molecular replacement using *Phaser* as implemented in the *PHENIX* package (McCoy *et al.*, 2007; Adams *et al.*, 2010). For the data set from the HigA2–DNA complex in space group $P3_121$, the structure of the C-terminal domain of the HigA2 antitoxin (S. Hadži, unpublished work) was used as search model, followed by a second search with the structure of a 17 bp DNA duplex. This DNA model was built with the *3DNA* program (van Dijk & Bonvin, 2009) assuming a B-conformation geometry and a global bend angle of 30°. For the HigA2–DNA complex in space group $P4_32_12$, a similar procedure was used except that the data were first corrected for anisotropic diffraction using an ellipsoidal truncation as implemented in the UCL Diffraction Anisotropy Server (Strong *et al.*, 2006).

Table 3

Oligonucleotides used for refinement.

Palindromic repeats are underlined.

No.	Sequence	Length	Ends	Resolution (Å)
1	GTACGCTTGGT <u>GCGTAC</u>	17	Blunt	2.3
2	ATACGCTTGGT <u>GCGTAC</u>	17	T/A	—
3	GTACGCTTGGT <u>GCGTAC</u>	17	C/G	30
4	TACGCTTGGT <u>GCGTAT</u>	16	T/AT	—
5	GTACGCTTGGT <u>GCGTACA</u>	18	Blunt	—
6	TGTACGCTTGGT <u>GCGTAC</u>	18	T/A	12
7	TGTACGCTTGGT <u>GCGTACA</u>	18	C/GC	9
8	TGTACGCTTGGT <u>GCGTACA</u>	19	Blunt	—
9	CTGTACGCTTGGT <u>GCGTAC</u>	19	C/G	3.5
10	TGCCATCTGTACGCTTGGT <u>GCGTACACTTCC</u>	31	T/A	3.5
11	CGCCATCTGTACGCTTGGT <u>GCGTACACTTCC</u>	31	C/G	3.3
12	TGCCATCTGTACGCTTGGT <u>GCGTACACTTCTTA</u>	33	Blunt	7
13	TGCCATCTGTACGCTTGGT <u>GCGTACACTTCTTA</u>	33	T/A	—
14	TGCCATCTGTACGCTTGGT <u>GCGTACACTTCTTAT</u>	35	Blunt	—
15	TGCCATCTGTACGCTTGGT <u>GCGTACACTTCTTAT</u>	35	T/A	—

The structure of HigBA2ΔHis in complex with a 31 bp DNA duplex was also determined by molecular replacement using *Phaser*, but using the solution of the HigA2–DNA complex in space group $P3_121$ and the structure of His–HigB2 (S. Hadži, unpublished work) as search models.

3. Results and discussion

3.1. Crystallization strategy

The *higBA2* operator sequence was determined by copper–phenanthroline footprinting and contains a 7 bp palindromic repeat with a five-base spacer at its centre (TGTACGCTTGGTGCGTACA; S. Hadži, unpublished work). In a first screening step we used a series of nine oligonucleotide sequences with different endings and lengths (Table 2). In all of these sequences the operator sequence is located centrally. The duplexes were between 17 and 31 bp in length and were either blunt-ended or ended with a single-base overhang to support the formation of a continuous helix throughout the crystal lattice. The lengths of the duplexes were chosen such that there is an integer number of helical turns in the duplex (21 or 31 base pairs), which supports the formation of continuous DNA helices. On the other hand, when the DNA molecules are bent the use of duplexes with a half-integer number of helical turns (17 or 26 base pairs) places less restriction on the orientation of the adjacent helices (Tan *et al.*, 2000) and therefore such duplexes were also considered.

In the initial screen, three protein constructs were used: HigA2 antitoxin, His–HigBA2 complex and HigBA2ΔHis complex. The antitoxin is a dimer in solution, while both of the HigBA2 complexes form heterotetramers (Hadži *et al.*, 2013). Using these proteins and the DNA duplexes, we prepared 27 different nucleoprotein complexes and subjected them to crystallization trials with various commercially available screens, resulting in >25 000 trials.

3.2. Crystals of HigA2–DNA complexes

In the initial screening with the HigA2 antitoxin several hits were observed with the blunt-ended 17 bp duplex (sequence 1

in Table 2). All crystals were morphologically identical but differed in size. The largest of these crystals, which grew in 0.1 M sodium citrate tribasic dihydrate pH 5.5, 16% (w/v) PEG 8000, was preliminary characterized at the PROXIMA1 beamline and diffracted to around 6 Å resolution.

Following our initial success with the 17 bp duplex, we designed a second set of oligonucleotides (Table 3, sequences 1–9) that were used to refine the search around the first hit. These oligonucleotides anneal to duplexes with different endings: blunt ends, sticky ends with A/T or C/G single-base

Figure 1
Crystals of HigA2 antitoxin and HigBA2 complexes with the operator. (a) HigA2 with a 17 bp blunt-ended duplex (Table 3, sequence 1). (b) HigA2 with an 18 bp duplex with C and GC overhangs (Table 3, sequence 7). (c) HigA2 with a 19 bp duplex with C/G overhangs (Table 3, sequence 9). (d) HigBA2 Δ His with a 31 bp duplex with T/A overhangs (Table 3, sequence 10). (e) HigBA2 with a 31 bp duplex with C/G overhangs (Table 3, sequence 11). (f) HigBA2 with a 33 bp blunt-ended duplex (Table 3, sequence 12). The black bar corresponds to 20 μ m and the scale is identical for all images.

overhangs and Watson–Crick pairing or T/AT or C/GC for triple-strand overhangs and Hoogsteen pairing. Crystals large enough to be harvested grew within a week in several crystallization conditions (for a full list of crystallization hits refer to Supplementary Table S1). Most hits were observed with the 17 bp duplex with blunt ends, the 18 bp duplex with C/CG overhangs and the 19 bp duplex with C/G single-base overhangs (sequences 1, 7 and 9, respectively, in Table 3). Typical

crystals formed with these DNA duplexes are shown in Figs. 1(a), 1(b) and 1(c), respectively. A few crystals were also observed with sequences 3 and 6 as listed in Table 3; however, they had poor diffraction power.

Most crystals that were tested from the various conditions and with the different DNA duplexes showed poor or no diffraction. However, crystals from ProPlex condition E9 [0.2 M sodium chloride, 0.1 M Na HEPES pH 7.5, 12% (w/v) PEG 8000] with the blunt-ended 17 bp duplex diffracted to 2.33 Å resolution after cryoprotection by adding 30% glycerol (Figs. 1a and 2a). These crystals belonged to space group $P3_22_1$, with unit-cell parameters $a = b = 94.0$, $c = 123.7$ Å. Data-collection statistics are given in Table 4.

Additionally, co-crystallization experiments using a 19 bp duplex with a C/G overhang resulted in a second crystal form (Fig. 1c). These crystals grow in ProPlex condition G10 with a high ionic strength (1.6 M sodium/potassium hydrogen phosphate pH 6.5; for cryoprotection the reservoir solution was supplemented with 40% glycerol). They belonged to space group $P4_32_12$. The unit cell is rather elongated, with unit-cell parameters $a = b = 61.2$, $c = 228.7$ Å. These crystals showed anisotropic diffraction that reached 3.46 Å in the c direction, while in the orthogonal directions diffraction was observed to only 5.8 Å resolution (Fig. 2b).

3.3. Crystals of HigBA2–DNA complexes

In the initial screening for toxin–antitoxin–operator crystals we used the same DNA sequences as for the HigA2 complexes (Table 2). A promising hit was found with sequence 9 (Table 2), which corresponds to a 31 bp duplex with single base-pair T/A overhangs. Crystals were only observed with the

(a)

(b)

(c)

Figure 2 Diffraction patterns of antitoxin–operator crystals of the trigonal $P3_22_1$ form (a) and the tetragonal $P4_32_12$ crystal form (b) and the HigBA2 Δ His–operator crystals (c).

Table 4

Data collection and processing.

Values in parentheses are for the outer shell.

	HigA2–DNA (17 bp blunt-ended)	HigA2–DNA (19 bp C/G overhang)	HigBA2ΔHis–DNA (31 bp C/G overhang)
Diffraction source	PROXIMA1, SOLEIL	PROXIMA1, SOLEIL	PROXIMA2, SOLEIL
Wavelength (Å)	0.97911	0.97911	0.98010
Temperature (K)	100	100	100
Detector	PILATUS	PILATUS	ADSC
Crystal-to-detector distance (mm)	509.7	600.8	429.0
Rotation range per image (°)	0.1	0.2	0.5
Total rotation range (°)	200	200	90
Space group	$P3_221$	$P4_32_12$	$P4_12_12$
a, b, c (Å)	94.0, 94.0, 123.7	61.2, 61.2, 228.7	113.6, 113.6, 121.1
α, β, γ (°)	90, 90, 120	90, 90, 90	90, 90, 90
Mosaicity (°)	0.092	0.406	0.218
Resolution range (Å)	49.26–2.33 (2.47–2.33)	47.73–3.46† (3.67–3.46)	48.30–3.30 (3.49–3.30)
Total No. of reflections	279349 (34557)	83827 (12710)	86951 (13618)
No. of unique reflections	27229 (4150)	6199 (926)	12408 (1921)
Completeness (%)	99.2 (94.9)	99.4 (97.3)	99.3 (98.6)
Multiplicity	10.3 (8.3)	13.5 (13.7)	7.0 (7.1)
$\langle I/\sigma(I) \rangle$	18.15 (2.02)	6.71 (1.42)‡	12.3 (2.0)
$R_{\text{r.i.m.}}$	0.078 (0.793)	0.443 (3.70)	0.187 (1.23)
Overall B factor from Wilson plot (Å ²)	54.1	82.5	95.8

† Diffraction was anisotropic and in the orthogonal directions it reached only 5.8 Å resolution. ‡ The $\langle I/\sigma(I) \rangle$ in the outer shell is lower owing to a spherical resolution cutoff which includes reflections in the weakly diffracting directions owing to anisotropy.

HigBA2ΔHis complex; the complex that included the affinity tag failed to produce any crystals.

Again, a second set of oligonucleotides was designed to refine the search (Table 3, sequences 10–15). Duplexes from these oligonucleotides are between 31 and 35 bp in length and are either blunt-ended or end with single-base overhangs that form A/T or C/G Watson–Crick pairs. In a number of different conditions we observed the formation of harvestable crystals that grew within a couple of weeks and contained sequences 10, 11 and 12 (Figs. 1*d*, 1*e* and 1*f*, respectively; for a full list of crystallization hits refer to Supplementary Table S2). Complexes with duplexes longer than 33 bp gave no or very few hits that were too small to be harvested. While most crystals had poor diffraction to around 13 Å or to 7 Å resolution at best, we found two conditions where crystals diffracted to around 3.5 Å resolution. The first was PEGRx condition H4 [15% (v/v) 2-propanol, 0.1 M sodium citrate tribasic dihydrate pH 5.0, 10% PEG 10 000; for cryoprotection the reservoir solution was supplemented with 20% glycerol]. Here, the crystal contained a 31 bp duplex with T/A overhangs. These crystals belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 113.6$, $c = 121.1$ Å. Unfortunately, the diffraction was again anisotropic: diffraction along the c axis was measured to 3.6 Å resolution, while in the orthogonal directions it was to less than 4.6 Å resolution. The second condition that gave diffraction-quality crystals was ProPlex condition C12 [0.2 M potassium iodide, 0.1 M MES pH 6.5, 25% (w/v) PEG 4000; for cryoprotection the reservoir solution was supplemented with 15% glycerol]. These crystals also contained a 31 bp duplex but ending with C/G overhangs. In terms of space group and unit-cell parameters these crystals are identical to the previous crystals. However, the diffraction quality was better: the diffraction showed no significant anisotropy and reached 3.3 Å resolution. Data-collection

statistics for crystals of HigBA2ΔHis in complex with the 31 bp duplex with C/G overhangs are given in Table 4.

3.4. Crystal packing

Structures of HigA2–DNA from both crystal forms were solved using molecular replacement. In all cases there is one molecule of the nucleoprotein complex in the asymmetric unit. Since refinement is still in progress, we will limit ourselves to discussion of the structure of the crystal lattice. A previously successful strategy for obtaining diffraction-quality crystals of protein–DNA complexes is trying to form a continuous DNA helix that runs throughout the crystal lattice (Anderson *et al.*, 1984; Tan *et al.*, 2000). Continuous DNA helices are indeed present in all three crystal forms reported here. As also observed previously, the use of different oligonucleotide sequences strongly affects the structure of the continuous helix and consequently the structure of the crystal lattice and the diffraction properties. When the antitoxin HigA2 co-crystallizes with a 19 bp duplex with C/G overhangs the crystals belonged to the tetragonal space group $P4_32_12$, while co-crystallization with a 17 bp duplex with blunt ends results in crystals belonging to the trigonal space group $P3_221$. In both cases the DNA molecule has the same bend angle of around 30°. Because blunt ends do not impose constraints in the orientation of the adjacent DNA molecule, these are rotated by 180° (the a direction) in the $P3_221$ crystal form (Fig. 3*a*). Moreover the helix is not straight, but each adjacent molecule is rotated by 30° in the b direction, again showing a zigzag shape. The axis of the continuous DNA helix is not parallel to the crystallographic axis. Rather, continuous DNA helices are rotated by 120° and stacked one upon another, giving rise to the 3_2 screw axis along the c direction. On the other hand, C/G overhangs impose constraints on the orientation of the adja-

Figure 3
Analysis of the crystal lattice structure of the antitoxin–operator complex in the trigonal $P3_21$ crystal form (a) and the tetragonal $P4_32_12$ crystal form (b). In the lower panels the view in the c direction is shown. The unit cell is shown in green.

cent molecule because of the formation of a base pair with the neighbouring molecule. In the continuous helix in the $P4_32_12$ crystal form each antitoxin is rotated relative to another by approximately 90° (Fig. 3b) and the helical axis coincides with the 4_3 screw axis. After about 100 base pairs the fifth antitoxin molecule has the same orientation as the first one, which gives rise to a rather long unit cell in the c direction. The continuous helices are stacked one upon another in parallel fashion along the a and b axis. This explains why diffraction is much better in the c direction compared with the orthogonal directions.

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