Expression, purification, crystallization and preliminary X-ray analysis of CttA, a putative cellulose-binding protein from *Ruminococcus flavefaciens*

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A number of anaerobic microorganisms produce multi-modular, multi-enzyme complexes termed cellosomes. These extracellular macromolecular nanomachines are designed for the efficient degradation of plant cell-wall carbohydrates to smaller sugars that are subsequently used as a source of carbon and energy. Cellulolytic strains from the rumens of mammals, such as *Ruminococcus flavefaciens*, have been shown to have one of the most complex cellosomal systems known. Cellosome assembly requires the binding of dockerin modules located in cellosomal enzymes to cohesin modules located in a macromolecular scaffolding protein. Over 220 genes encoding dockerin-containing proteins have been identified in the *R. flavefaciens* genome. The dockerin-containing enzymes can be incorporated into the primary scaffoldin (ScaA), which in turn can bind to adaptor scaffoldins (ScaB or ScaC) and subsequently to anchoring scaffoldin (ScaE), thereby attaching the whole complex to the cell surface. However, unlike other cellosomes such as that from *Clostridium thermocellum*, the *Ruminococcus* species lack a specific carbohydrate-binding module (CBM) on ScaA which recruits the entire complex onto the surface of the substrate. Instead, a cellulose-binding protein, CttA, comprising two putative tandem novel carbohydrate-binding modules and a C-terminal X-dockerin module, which can bind to the cohesin of ScaE, may mediate the attachment of bacterial cells to cellulose. Here, the expression, purification and crystallization of the carbohydrate-binding modular part of the CttA from *R. flavefaciens* are described. X-ray data have been collected to resolutions of 3.23 and to 1.61 Å in space groups $P3_121$ or $P3_221$ and $P2_1$, respectively. The structure was phased using bound iodide from the crystallization buffer by SAD experiments.

1. Introduction

Anaerobic bacteria, in particularly those that colonize the rumens of mammals, such as *Ruminococcus flavefaciens*, organize cellulases and hemicellulases into a large megadalton, multi-enzyme complex (molecular weight >3 MDa) termed the cellosome (Bayer *et al.*, 2004). These highly intricate nanomachines play an integral role in the efficient deconstruction of plant cell-wall carbohydrates, thus contributing to carbon turnover in nature. *R. flavefaciens* strains are known to vary widely in their cellosome organization and plant cell wall-degrading activities (Dehority & Scott, 1967; Berg Miller *et al.*, 2009). For example, *R. flavefaciens* strains

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FD-1, 17 and 0007c present subtle differences in the organization of the scaffoldin gene cluster which lead to significant differences in the topologies of the secreted cellulosomes (Dassa *et al.*, 2014; Rincon *et al.*, 2005; Jindou *et al.*, 2006). Cellulosome assembly depends on the binding of dockerin modules, which are usually located at the C-terminus of the cellulosomal enzymes, to cohesin modules located in a primary scaffoldin (Fontes & Gilbert, 2010). In *R. flavefaciens*, dockerin-containing enzymes are incorporated into the primary multimodular scaffoldin (ScaA), which in turn can bind to adaptor scaffoldins (ScaB or ScaC) that subsequently bind to the anchoring cell surface scaffoldin (ScaE). This complex mechanism of cellulosome assembly also promotes the attachment of the whole complex to the cell surface. However, unlike other cellulosomes such as those from *Clostridium* species, *R. flavefaciens* scaffoldins lack a cellulose-specific carbohydrate-binding module (CBM) which targets the large multi-enzyme complex onto its primary substrate. Instead, *R. flavefaciens* expresses a separate cellulose-binding protein, CttA, comprising two putative tandem carbohydrate-binding modules followed by a Thr/Ser-rich linker region and a C-terminal X-dockerin module which binds the protein to the cell surface scaffoldin ScaE (Fig. 1). Thus, the function of CttA is to bind the entire bacterial cell to cellulose (Rincon *et al.*, 2007; Flint *et al.*, 2008). BLAST analysis shows that CttA has no structural homologues, and so far only six CttAs, all from different *R. flavefaciens* strains, have been identified (Altschul *et al.*, 1990; Supplementary Fig. S1 and Table S1). These CttAs are 699–838 amino-acid residues in length, with the carbohydrate-binding moiety comprising two putative CBMs of about 180 amino-acid residues each (Fig. 1). In order

Figure 1

Schematic diagram displaying the modular architecture of the full-length *R. flavefaciens* CttA. The two regions that contain the putative CBMs are shown in green, corresponding to the region 32–425 of the 803 amino-acid residues of CttA1 and the region 30–415 of the 736 amino-acid residues of CttA3. TS-rich linker, threonine/serine-rich linker sequence; X, X module; Doc, dockerin module. The C-terminal X-dockerin module is depicted as binding to the cohesin of the ScaE anchoring scaffoldin located on the cell surface of the bacterium.

Table 1

Macromolecule-production information.

Cloning vector	pET-28a
Expression vector	pET-28a
Expression host	<i>E. coli</i> BL21
CttA1†	MGSSHHHHHSSGLVPRGSHMASAENDAVQIVGNTVQDGAAGQ-TVSLLESLLYIPANKTESTWNVTGSAALAKMVGTKGSINNA-KILDLVKYVPANYREAAKAALEEVVLVNPVTVEVTADKNI-VLKATVSQPNFNGNWNTPGKALSDLAAYNAPELNAVDF-SSVKVGGDLVVTIKTSKLDEGTTVPVEVVKYKADGDIYAG-DFAKFANDKLTLENI AKKAIENDVAAQYAKEATDKFLAK-TALIRDKINKAEKALDQALTKTADYKISEAADAANKFLA-KKNINKKVPSSAAEVAKEKTKKYDYAAVKKASAKADIQI-TVEQLGAFVDSLTSKTLTDSEGNFATAFDINLNGKATL-VGAFEDAEADVQKWEKGYAYVGSYKLTGTVDFTGK-TADTASVDVQIERILVTE
CttA3‡	MGSSHHHHHSSGLVPRGSHMASESNDVQTATAQAESSSK-ITLDDLLYIPANKTVSDWNQLVWAKLATRTQDGTFLNTE-LAKEAVEHAGQFKDVADYALNELIIPNGVKYQITDNDVV-ITGHISEPDFNRNLSLTSGNALAIQAENYKAPNLTKIDFS-DISVEGDFKITFETSKLGAGTEVP IKFEYKTTADNKVYAA-GQLFDLALDTMAKVKAKGDAALEKEIPAQFVDKAKADYAE-KIKNVTDKIEKAKRNFEKALKAEKSGEYANVAGVIKANT-WLKNHNINKQIPATATDIAAKSIVTKAYDQLLNDFSQEHD-INIATAADLGKFADSLNKGSLAKAVANGEATFKDAEQAE-VKAYVESLGYAFVDSYKVKTAQVDFSGINTTDAGSVAVKI-ERVLVTN

† WP_009986661.1 or embL_CAK18897.1, corresponding to residues 31–425 of the 803 residues of the full-length protein. Extra residues from the cloning artefact, including the N-terminal His₆ tag, are underlined. ‡ WP_028518574.1 or embL_CAO00731.1, corresponding to residues 30–415 of the 736 residues of the full-length protein. Extra residues from the cloning artefact, including the N-terminal His₆ tag, are underlined.

to gain insight into the structural properties that govern ligand recognition by this cellulose-binding protein, we aim to determine the crystal structures of the putative CBMs of CttA from *R. flavefaciens* strain FD-1, termed CttA1, and from strain C94 isolated from Holstein cows, termed CttA3 (Supplementary Fig. S1). Here, we describe the overproduction, purification, crystallization and preliminary X-ray analysis of CttA from *R. flavefaciens*.

Figure 2

Coomassie Brilliant Blue-stained 12% SDS-PAGE gel evaluation of protein purity for CttA1 (a) and CttA3 (b). Lane 1, low-molecular-weight protein marker (NZYTech, Portugal; labelled in kDa); lane 2, CttA1 (a) or CttA3 (b).

2. Materials and methods

2.1. Macromolecule production

The genes encoding CttA1 and CttA3 were synthesized *in vitro* (NZYTech, Portugal) with codon usage optimized for expression in *Escherichia coli*. The synthesized genes, containing engineered NheI and XhoI restriction sites at the 5' and 3' ends, respectively, were used for subsequent subcloning into the pET-28a vector (Novagen), generating *pcttA1* and *pcttA3*. The encoded recombinant proteins contained an N-terminal His₆ tag (Table 1). Plasmids *pcttA1* and *pcttA3* were used to transform *E. coli* BL21 cells, which were cultured in Luria–Bertani broth at 310 K to mid-exponential phase ($OD_{600\text{ nm}} = 0.6$). Recombinant protein overproduction was then induced by adding isopropyl β -D-1-thiogalactopyranoside (1 mM final concentration) and incubation for a further 16 h at 298 K. The cells were collected after centrifugation and the pellets were resuspended in 50 mM Na HEPES pH 7.5, 10 mM imidazole, 1 M NaCl, 5 mM CaCl₂ buffer. The cells were lysed by sonication. The His₆-tagged recombinant proteins were purified from cell-free extracts by immobilized metal-ion affinity chromatography (IMAC) as described previously (Najmudin *et al.*, 2006). Purified CttA1 and CttA3 were buffer-

exchanged into 50 mM Na HEPES pH 7.5 buffer containing 200 mM NaCl and 5 mM CaCl₂ and subjected to gel filtration using a HiLoad 16/60 Superdex 75 column (GE Healthcare) at a flow rate of 1 ml min⁻¹. CttA1 and CttA3 were concentrated using an Amicon 10 kDa molecular-mass centrifugal concentrator and washed three times with water (Sigma) containing 0.5 mM CaCl₂. Recombinant CttA1 and CttA3 with the engineered N-terminal His₆ tag have calculated molecular masses of 44.8 and 44.5 kDa, respectively. The protein concentration was estimated using molar extinction coefficients (ϵ) of 38 850 and 34 380 M⁻¹ cm⁻¹ at 280 nm for CttA1 and CttA3, respectively, with a Thermo Scientific NanoDrop 2000c. Protein homogeneity was shown by SDS–PAGE (Fig. 2).

2.2. Crystallization

Crystallization conditions for CttA1 were screened by hanging-drop vapour diffusion using the commercial kits Crystal Screen, Crystal Screen 2, PEG/Ion and PEG/Ion 2 (Hampton Research, California, USA). CttA1 crystals were obtained in various conditions within one month. Crystallization conditions for CttA3 were screened by the sitting-

Figure 3

(a) Crystals of CttA3 obtained by the sitting-drop vapour-diffusion method with PEG/Ion condition No. 10 [0.2 M sodium iodide, 20% (w/v) polyethylene glycol 3350; data set x9 in Table 3]. (b, c, d) Crystals of CttA3 obtained by the hanging-drop vapour-diffusion method using Additive Screen: (b) 0.2 M sodium iodide, 20% (w/v) polyethylene glycol 3350 + 0.1 M ethylenediaminetetraacetic acid disodium salt dehydrate (data set x1), (c) 0.2 M sodium iodide, 20% (w/v) polyethylene glycol 3350 + 10 mM calcium chloride dehydrate (data set x2) and (d) 0.2 M sodium iodide, 20% (w/v) polyethylene glycol 3350 + 10 mM sodium bromide (data set x6). Some of the crystals grown in the condition for (d) were soaked for at least half an hour in cryoprotectant buffer with 10 mM CdI₂ added to it before cryocooling and testing at SOLEIL (data set x8_CdI₂). The scale bar represents 0.1 mm.

Table 2
Crystallization.

Protein	CttA3	CttA3
Method	Sitting-drop vapour diffusion (using the commercial kits Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2 and JBScreen 1–4)	Hanging-drop vapour diffusion (using Additive Screen)
Plate type	MRC 96-well 2-drop sitting-drop crystallization plate (Molecular Dimensions)	Greiner 24-well hanging-drop crystallization plate (Molecular Dimensions)
Temperature (K)	292	292
Protein concentration (mg ml ⁻¹)	50, 30, 15	50, 30, 50 + 14 mM 1,4-β-D-cellohexaose
Buffer composition of protein solution	Deionized water (Sigma), 0.5 mM CaCl ₂	Deionized water (Sigma), 0.5 mM CaCl ₂
Composition of reservoir solution	0.2 M sodium iodide, 20%(w/v) polyethylene glycol 3350 (Fig. 3a; data set x9)	0.2 M sodium iodide, 20%(w/v) polyethylene glycol 3350 + 0.01 M ethylenediaminetetraacetic acid disodium salt dihydrate (Fig. 3b; data set x1) 0.2 M sodium iodide, 20%(w/v) polyethylene glycol 3350 + 0.01 M calcium chloride dihydrate (Fig. 3c; data set x2) 0.2 M sodium iodide, 20%(w/v) polyethylene glycol 3350 + 0.01 M sodium bromide (Fig. 3d; data set x6)
Volume and ratio of drop	0.7 µl protein solution, 0.7 µl reservoir solution	1 µl protein solution, 1 µl reservoir solution
Volume of reservoir (µl)	30	600

Table 3
Data-collection and processing statistics for CttA3.

Values in parentheses are for the outer shell.

Data set	CttA3 x9	CttA3 x1_SAD	CttA3 x1_hi	CttA3 x2_SAD	CttA3 x6	CttA3 x8_CdI ₂
Diffraction source	BM14, ESRF	I02, Diamond	I02, Diamond	I02, Diamond	I02, Diamond	PROXIMA 1, SOLEIL
Wavelength (Å)	0.95373	2.0	0.97974	2.19997	0.97949	
Temperature (K)	100	100	100	100	100	100
Detector	MAR 225 CCD	PILATUS 6M, Dectris	PILATUS 6M, Dectris	PILATUS 6M, Dectris	PILATUS 6M, Dectris	PILATUS 6M, Dectris
Rotation range per image (°)	1	0.2	0.2	0.2	0.2	0.2
Total rotation range (°)	100	360	360	360	360	360
Exposure time per image (s)	1	0.2	0.2	0.2	0.2	0.2
Space group	<i>P</i> ₃ ₁ 21 or <i>P</i> ₃ ₂ 21	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	98.88, 98.88, 180.80	45.18, 43.53, 78.41	45.14, 43.49, 78.37	44.77, 40.81, 75.93	45.02, 42.73, 78.35	44.80, 43.77, 77.89
α , β , γ (°)	90.00, 90.00, 120.00	90.00, 104.19, 90.00	90.00, 104.19, 90.00	90.00, 103.72, 90.00	90.00, 104.14, 90.00	90.00, 104.16, 90.00
Mosaicity (°)	1.41	0.2	0.2	0.31	0.25	0.4
Matthews coefficient† (Å ³ Da ⁻¹)	2.99 or 1.99	1.81	1.81	1.81	1.81	1.81
Solvent content (%)	59 or 38	32	32	32	32	32
Molecules in asymmetric unit	2 or 3	1	1	1	1	1
Resolution range (Å)	62.16–3.23 (3.49–3.23)	43.80–2.11 (2.17–2.11)	43.76–1.87 (1.92–1.87)	43.49–2.97 (3.05–2.97)	37.99–1.61 (1.65–1.61)	37.98–2.60 (2.74–2.60)
Total No. of reflections	97100 (19634)	78806 (461)	165500 (12234)	34527 (2229)	248824 (17871)	174304 (11456)
No. of unique reflections	17001 (3429)	12831 (163)	24410 (1759)	5596 (393)	36894 (2662)	17476 (1298)
Completeness (%)	99.1 (99.4)	74.8 (12.9)	99.0 (97.9)	98.8 (96.2)	98.1 (96.6)	99.1 (99.0)
Multiplicity	5.7 (5.7)	6.1 (2.8)	6.8 (7.0)	6.2 (5.7)	6.7 (6.7)	9.8 (8.8)
CC _{1/2} ‡	0.991 (0.497)	0.998 (0.977)	0.999 (0.877)	0.986 (0.867)	1.000 (0.846)	1.000 (0.691)
$\langle I/\sigma(I) \rangle$	7.9 (1.3)	28.5 (7.4)	19.2 (3.1)	9.2 (2.0)	26.6 (2.8)	19.1 (1.6)
R _{p.i.m.} § (%)	5.6 (65.5)	2.2 (8.2)	2.2 (22.9)	5.8 (26.6)	1.4 (31.0)	8.5 (151.6)

† Matthews (1968). ‡ The correlation coefficient between intensities from random half data sets (Diederichs & Karplus, 2013). § R_{p.i.m.} = $\sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the average of symmetry-related observations of a unique reflection.

drop vapour-diffusion method using the commercial kits Crystal Screen, Crystal Screen 2, PEG/Ion and PEG/Ion 2 (Hampton Research, California, USA) and JBScreens 1–4 (Jena Bioscience, Germany) with an Oryx8 robotic nanodrop dispensing system (Douglas Instruments). Crystals of CttA3 grew in several conditions, but most easily in PEG/Ion condition No. 10: 0.2 M sodium iodide, 20%(w/v) polyethylene glycol 3350 (Fig. 3a). Additive Screen from Hampton Research (California, USA) was set up around this condition using the hanging-drop vapour-diffusion method. These crystals also took at least a month to grow to their maximum size. The conditions that gave diffracting crystals are given in

Table 2 and shown in Fig. 3. The crystals were vitrified in liquid nitrogen after soaking in cryoprotectant [30%(v/v) glycerol (and 10 mM of additive for crystals grown in the optimization screens) added to the crystallization buffer] for a few seconds.

2.3. Data collection and processing

The crystals of CttA1 were tested on beamlines BM14, ID14-4 and BM30 at ESRF, France and I03 at Diamond Light Source, England. Since the CttA1 crystals were unable to generate good-quality data, all subsequent testing was carried out on CttA3 crystals on beamlines BM14 at ESRF, I02 at

Diamond) and PROXIMA 1 at SOLEIL, France (Fig. 2, Table 2). Initially, these crystals were tested on beamline BM14 at ESRF, with the best data being obtained to a resolution of 3.23 Å (Table 3) for crystals grown in 0.2 M NaI. Further optimization screens were set up based on this condition to exploit the potential weak signal from the iodides (present in the crystallization buffer) for phasing, and redundant data were collected at long wavelengths on I02 at Diamond. Crystals grown in optimization screens with additives to introduce anomalously scattering elements (such as CdI₂) that may aid in phase determination were also tested at various wavelengths on PROXIMA 1 at SOLEIL. All data sets were processed using *iMosflm* (Battye *et al.*, 2011) or *XDS* (Kabsch, 2010) with *fast_dp* (Winter, 2010) and *xia2* (Winter *et al.*, 2013) or via the command-line interface *xdsme* (<https://code.google.com/p/xdsme/>) and *AIMLESS* (Evans, 2006) from the CCP4 suite (Collaborative Computational Project, Number 4, 1994; Winn *et al.*, 2011). Data-collection statistics (as reported by *AIMLESS*) are given in Table 3.

3. Results and discussion

3.1. Crystal optimization

Commercially available sparse-matrix screens were used to manually set up 2 µl (1 µl protein solution plus 1 µl reservoir solution) hanging-drop vapour-diffusion experiments at 292 K at three different concentrations of CttA1: 15, 30 and 50 mg ml⁻¹. Crystals were obtained in four different conditions: (i) 0.2 M ammonium sulfate, 30% PEG 8000, (ii) 0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate pH 4.6, 30% PEG MME 2000, (iii) 0.2 M sodium tartrate dibasic dehydrate, 20% PEG 3350 and (iv) 0.2 M potassium sodium tartrate tetrahydrate, 20% PEG 3350. A series of optimization experiments were set up varying the counterion concentration from 100 to 340 mM in steps of 10 mM and varying the PEG/PEG MME concentration from 16 to 35%. The crystals grown in ammonium sulfate all turned out to be of salts, whereas those grown in tartrate diffracted very weakly.

Since we had difficulty in obtaining good diffracting crystals of CttA1 from strain FD-1, we turned our attention to the homologue CttA3 from strain C94 (with 39% sequence identity). This time we were able to screen hundreds of crystallization conditions using more commercially available sparse-matrix screens and automatically setting up drops of 1.4 µl (0.7 µl protein solution plus 0.7 µl reservoir solution) by sitting-drop vapour-diffusion experiments at 292 K at three different concentrations of CttA3: 15, 30 and 50 mg ml⁻¹. Long plank-like crystals that diffracted to 3.23 Å resolution were obtained in 0.2 M sodium iodide, 20% (w/v) polyethylene glycol 3350 (Fig. 3a; data set x9). Similar crystals were also obtained in conditions from the PEG/Ion screen in which the sodium iodide was replaced by sodium fluoride, potassium fluoride, ammonium fluoride, lithium chloride and potassium fluoride. Since CttA3 has only one integral methionine, it is unlikely that we would be able to solve the structure by using selenomethionine derivatives for phasing; therefore, we

decided to set up optimization screens around the sodium iodide condition using Additive Screen (Hampton Research, USA). Since we were not able to obtain crystals at the lower protein concentration of 15 mg ml⁻¹, we used 50 mg ml⁻¹ CttA3 with 14 mM 1,4-β-D-cellohexaose as one of the three crystallization drops per condition set up in hanging-drop vapour-diffusion experiments. Adding potential ligands often stabilizes the protein and can yield better crystals. The combination of using the substrate and additives gave more robust crystals with a different morphology and improved the diffraction by 1.6 Å (Figs. 3b, 3c and 3d, Table 3). Thus, we were able to obtain crystals that not only diffracted to a much higher resolution but gave phases from the bound iodide ions that led to the eventual structure determination.

3.2. Structure determination

The CttA3 structure was determined using the anomalous signal from iodide ions by a single-wavelength anomalous dispersion experiment. Data set x1_SAD (see Table 3) processed by *fast_dp* was used to identify five heavy-atom sites using the *SHELX* suite (Sheldrick, 2008) via the *HKL2MAP* graphical interface (Pape & Schneider, 2004). Phasing and heavy-atom completion using *Phaser* (McCoy *et al.*, 2004, 2007) extended the number of sites to five iodide sites, a further site subsequently found to correspond to the SD atom of Met190 and four further sites interpreted as ordered Cl ions (or partially occupied iodide ions). These sites were then used to calculate phases using *Phaser* in SAD mode from the CCP4 suite (Winn *et al.*, 2011) followed by density improvement using *Parrot* (Zhang *et al.*, 1997). The quality of the electron-density maps allowed the identification of several secondary-structure elements. Modelling these elements and using them as input to *Phaser*, along with the previously determined sites, followed by density improvement permitted a significant improvement of the electron-density map. Automatic model building using *Buccaneer* (Cowtan, 2006, 2008) successfully placed 292 out of 408 residues with an *R* and *R*_{free} of 26.85 and 29.6%, respectively, to a resolution limit of 2.11 Å [produced by *REFMAC5* (Murshudov *et al.*, 2011) via the *Buccaneer* pipeline]. Use of this model in *Phaser*, giving no *a priori* information on the heavy-atom sites, allowed *Phaser* to determine a complete list of heavy-atom sites in agreement with those originally produced by *SHELXD*, hence validating the initial model. The model was corrected manually using *Coot* (Emsley *et al.*, 2010), and refinement and analysis are in progress to 1.61 Å resolution using data set x6 (see Table 3). The data set referred to as x8_CdI₂ (Table 3) showed weaker anomalous signal, with only a single site in common with the x1_SAD data, and was not used in the phasing procedure. The protein structure was shown to be identical to that based on the x1_SAD data. Data set x9 (Table 3) gave a possible solution (trimer) in space group *P*3₁21 with a very low, but potentially meaningful, TFZ of 6–7 and an LLG value of 158, using *Phaser* with the x1_SAD model. Further analysis is ongoing for these data.

4. Related literature

The following references are cited in the Supporting Information for this article: Tamura *et al.* (2007).

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References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). *J. Mol. Biol.* **215**, 403–410.
- Battye, T. G. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. W. (2011). *Acta Cryst.* **D67**, 271–281.
- Bayer, E. A., Belaich, J.-P., Shoham, Y. & Lamed, R. (2004). *Annu. Rev. Microbiol.* **58**, 521–554.
- Berg Miller, M. E. *et al.* (2009). *PLoS One*, **4**, e6650.
- Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* **D50**, 760–763.
- Cowtan, K. (2006). *Acta Cryst.* **D62**, 1002–1011.
- Cowtan, K. (2008). *Acta Cryst.* **D64**, 83–89.
- Dassa, B., Borovok, I., Ruimy-Israeli, V., Lamed, R., Flint, H. J., Duncan, S. H., Henrissat, B., Coutinho, P., Morrison, M., Mosoni, P., Yeoman, C. J., White, B. A. & Bayer, E. A. (2014). *PLoS One*, **9**, e99221.
- Dehority, B. A. & Scott, H. W. (1967). *J. Dairy Sci.* **50**, 1136–1141.
- Diederichs, K. & Karplus, P. A. (2013). *Acta Cryst.* **D69**, 1215–1222.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Evans, P. (2006). *Acta Cryst.* **D62**, 72–82.
- Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R. & White, B. A. (2008). *Nature Rev. Microbiol.* **6**, 121–131.
- Fontes, C. M. G. A. & Gilbert, H. J. (2010). *Annu. Rev. Biochem.* **79**, 655–681.
- Jindou, S., Borovok, I., Rincon, M. T., Flint, H. J., Antonopoulos, D. A., Berg, M. E., White, B. A., Bayer, E. A. & Lamed, R. (2006). *J. Bacteriol.* **188**, 7971–7976.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- McCoy, A. J., Storoni, L. C. & Read, R. J. (2004). *Acta Cryst.* **D60**, 1220–1228.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Najmudin, S., Guerreiro, C. I. P. D., Carvalho, A. L., Prates, J. A. M., Correia, M. A. S., Alves, V. D., Ferreira, L. M. A., Romão, M. J., Gilbert, H. J., Bolam, D. N. & Fontes, C. M. G. A. (2006). *J. Biol. Chem.* **281**, 8815–8828.
- Pape, T. & Schneider, T. R. (2004). *J. Appl. Cryst.* **37**, 843–844.
- Rincon, M. T., Cepeljnik, T., Martin, J. C., Barak, Y., Lamed, R., Bayer, E. A. & Flint, H. J. (2007). *J. Bacteriol.* **189**, 4774–4783.
- Rincon, M. T., Cepeljnik, T., Martin, J. C., Lamed, R., Barak, Y., Bayer, E. A. & Flint, H. J. (2005). *J. Bacteriol.* **187**, 7569–7578.
- Sheldrick, G. M. (2008). *Acta Cryst.* **A64**, 112–122.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). *Mol. Biol. Evol.* **24**, 1596–1599.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Winter, G. (2010). *J. Appl. Cryst.* **43**, 186–190.
- Winter, G., Lobley, C. M. C. & Prince, S. M. (2013). *Acta Cryst.* **D69**, 1260–1273.
- Zhang, K. Y. J., Cowtan, K. & Main, P. (1997). *Methods Enzymol.* **277**, 53–64.