Crystallization and X-ray analysis of the extracellular adhesion domain of *Helicobacter pylori* adhesin A: the significance of the cation composition in the crystallization precipitant

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Adherence to host cells is a crucial step in the process of bacterial infection, which is usually mediated by a number of outer membrane proteins identified as adhesins. *Helicobacter pylori* adhesin A (HpaA) is a member of the adhesin family that mediates the adherence of *Helicobacter pylori* to gastric epithelial cells, and consequently assists the bacteria in becoming a life-long colonizer of the human stomach. In this study, two constructs were made for the production of truncated HpaA proteins comprising residues 31–260 and 53–260, respectively. The products of both constructs were crystallized, but only the protein from the shorter construct (residues 53–260) formed crystals that were capable of diffraction. In the subsequent optimization trials, crystals in different forms were unexpectedly obtained by using lithium sulfate and ammonium sulfate as the precipitant. An X-ray data set was collected to 1.95 Å resolution on beamline BL18U1 at SSRF using a crystal grown with 1.92 M lithium sulfate, which belonged to space group $P6_5$ with unit-cell parameters $a = b = 95.42$, $c = 54.72$ Å, $\gamma = 120^\circ$, while another crystal grown with 1.9 M ammonium sulfate diffracted to 2.60 Å resolution and the collected data set was indexed in space group $P2_12_12$, with unit-cell parameters $a = 121.01$, $b = 190.56$, $c = 106.31$ Å. The collection of diffraction data has established a solid basis for structure determination.

1. Introduction

Helicobacter pylori is a ubiquitous pathogenic bacterium that infects roughly half of the global population (Goh *et al.*, 2011); it is aetiologically associated with a variety of gastroduodenal diseases such as gastritis, peptic ulcer and gastric atrophy. Persistent infection by *H. pylori* increases the risk of developing gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma (Hardbower *et al.*, 2014). Moreover, the presence of the bacterium has recently been reported to have a possible correlation with the development of many extra-gastric diseases, such as idiopathic thrombocytopenic purpura and iron-deficiency anaemia (Goni & Franceschi, 2016).

Three steps are usually involved in *H. pylori* infection: (i) ingestion by the host and survival in the acidic stomach, (ii) movement through a thick layer of mucus and (iii) adherence to the gastric epithelium and establishment of colonization (Kao *et al.*, 2016). In this process, adherence is crucial for successful colonization and persistent infection by providing

Table 1

Macromolecule-production information.

Protein	HpaA ₃₁	HpaA ₅₃
Source organism	<i>H. pylori</i> NCTC 26695	<i>H. pylori</i> NCTC 26695
DNA source	Genome	Genome
Forward primer†	GGAATTCCATATGCATATTATTGAAACCAAT- GAAGTCG	GGAATTCCATATGGCGTTAGATGAAAAGATTTTGC
Reverse primer‡	CCGCTCGAGTCGGTTTCTTTTGCTTTTA	CCGCTCGAGTCGGTTTCTTTTGCTTTTA
Cloning vector	pET-22b(+)	pET-22b(+)
Expression vector	pET-22b(+)	pET-22b(+)
Expression host	<i>E. coli</i> BL21(DE3)	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced§	MHIETNEVALKLNYPASEKVQALDEKILLRPAFQYSDNIAKEYENKF- KNQTKLKEQILQNQGYKVISVDSSDKDDLFSFSQKKEGYLAVAMNGEI- VLRPDPKRTIQKKSEPGLLFSTGLDKMEGVLPAGFVKVTILEPMSGE- SLDSFTMDLSELDIQEKFLKTTSSSHSGGLVSTMVKGTDNSNDAIKSA- LNKIFANIMQEIDKKLTQKNLESYQKDAKELGKGRNRLLEHHHHH	MALDEKILLRPAFQYSDNIAKEYENKFKNQTKLKEQILQNQGYKVISV- DSSDKDDLFSFSQKKEGYLAVAMNGEIVLRPDPKRTIQKKSEPGLLFST- GLDKMEGVLPAGFVKVTILEPMSGESLDSFTMDLSELDIQEKFLKTT- HSSHSGGLVSTMVKGTDNSNDAIKSALNKIFANIMQEIDKKLTQKNLE- SYQKDAKELGKGRNRLLEHHHHH

† The NdeI restriction site is underlined. ‡ The XhoI restriction site is underlined. § Non-native residues of HpaA originating from the expression vector are underlined.

protection for the bacteria against eradication from the host stomach by gastric peristalsis and mucus turnover. Notably, only the colonizing bacteria, which comprise only ~2% of the total bacteria found on the gastric surface, are able to cause host damage (Marcus & Scott, 2016; Dunne *et al.*, 2014).

The localized association of *H. pylori* with gastric epithelial cells has been attributed to selective binding of the bacteria to glycoproteins and glycolipids distributed on the host cell surface (Ångström *et al.*, 1998; Saitoh *et al.*, 1991; Lingwood *et al.*, 1992; Borén *et al.*, 1993). A number of outer membrane proteins in *H. pylori* have been identified as adhesins, including blood group antigen-binding adhesin (BabA), sialic acid-binding adhesin (SabA) and adherence-associated lipoproteins A and B (AlpA and AlpB). *H. pylori* adhesin A (HpaA), also referred to as neuraminylactose-binding haemagglutinin (NLBH), is an uncharacterized adhesin that seems to be essential for bacterial colonization in the mouse-adapted *H. pylori* Sydney strain 1 (SS1; Carlsohn *et al.*, 2006). This protein contains a predicted N-terminal transmembrane domain (residues 1–27) that functions as an anchor in attaching to the flagellar sheath and/or the outer membrane of *H. pylori* (Evans *et al.*, 1993; Jones *et al.*, 1997), and an extracellular adhesion domain (residues 28–260). To date, the mechanism of bacterial adhesion mediated by HpaA is poorly understood, although a few reports have predicted sialoglycoconjugates containing an *N*-acetylneuraminyl- α (2–3)-lactose group as possible receptors based on the prediction of a sialic acid-binding motif in the HpaA sequence (residues 134–139; Evans *et al.*, 1993; Simon *et al.*, 1997). Other investigators, however, identified another putative glycolipid-binding motif (residues 139–153) that may let HpaA recognize glycolipid receptors (Fantini *et al.*, 2006). It should be noted that all of these predictions were made merely from sequence comparison, secondary-structure prediction and tertiary-structure modelling rather than from an experimentally determined structure, which is still unavailable to date.

To date, crystal structures of two *H. pylori* adhesins, BabA and SabA, have been determined; these proteins share only 26% amino-acid sequence identity but have similar folds (PDB entry 4zh0, Pang *et al.*, 2014; PDB entry 4o5j, Hage *et al.*, 2015). HpaA shows even lower sequence homology to other

adhesins, implying that the protein probably recognizes a unique receptor, which however is difficult to identify without structural information. In this context, it becomes necessary to determine the crystal structure of this protein in order to understand the molecular mechanism of specific adhesin–receptor interaction and the pathological role played by HpaA in the process of *H. pylori* infection.

Here, we report the expression, purification, crystallization and preliminary X-ray crystallographic analysis of the extracellular adhesion domain of HpaA. The X-ray data collected to 1.95 Å resolution have established a solid basis for structural studies. Interestingly, crystallization of HpaA seemed to be rather sensitive to the cation composition of the precipitant, which gave rise to two different crystal forms using lithium sulfate and ammonium sulfate. We thus suppose that the results described here may serve as an unusual example highlighting the significance of cation choice in protein crystallization.

2. Materials and methods

2.1. Macromolecule production

The nucleotide sequence encoding the extracellular domain of HpaA (residues 31–260) was amplified from the genomic DNA of *H. pylori* NCTC 26695 using the PCR technique with cycle conditions consisting of initial denaturation at 367 K for 10 min followed by 35 cycles of 367 K for 40 s, annealing at 328.5 K for 30 s and extension at 345 K for 1 min, and final extension at 345 K for 5 min. The expression constructs covering residues 31–260 (named HpaA₃₁) and 53–260 (named HpaA₅₃) were constructed to produce C-terminally His-tagged proteins. The resultant constructs bearing an NdeI restriction site at the 5' end and a XhoI restriction site at the 3' end were digested with both restriction enzymes and ligated into similarly digested pET-22b(+) (Novagen) before validation by nucleotide sequencing (Sangon Biotech, People's Republic of China). More detailed information regarding the plasmid construction is given in Table 1.

The recombinant plasmids were subsequently transformed into *Escherichia coli* host strain BL21(DE3). The bacteria

Table 2
Crystallization of HpaA₅₃.

Method	Hanging drop	Sitting drop
Plate type	16-well plates	48-well plates
Temperature (K)	296	296
Protein concentration (mg ml ⁻¹)	20	20
Buffer composition of protein solution	150 mM NaCl, 20 mM Tris pH 8.0	150 mM NaCl, 20 mM Tris pH 8.0
Composition of reservoir solution	0.1 M HEPES pH 8.0, 1.9 M (NH ₄) ₂ SO ₄ , 5%(v/v) glycerol	0.1 M HEPES pH 7.0, 1.92 M Li ₂ SO ₄
Volume and ratio of drop	2 µl, 1:1	2 µl, 1:1
Volume of reservoir (µl)	500	100

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Protein crystals	HpaA ₅₃ [grown in (NH ₄) ₂ SO ₄]	HpaA ₅₃ (grown in Li ₂ SO ₄)
Diffraction source	BL17U1, SSRF	BL18U1, SSRF
Wavelength (Å)	0.9791	0.97776
Temperature (K)	100	100
Detector	ADSC Q315R	PILATUS 6M
Crystal-to-detector distance (mm)	300	350
Rotation range per image (°)	1.0	1.0
Total rotation range (°)	360	360
Exposure time per image (s)	0.5	0.5
Space group	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 6 ₅
<i>a</i> , <i>b</i> , <i>c</i> (Å)	121.01, 190.56, 106.31	95.42, 95.42, 54.72
α , β , γ (°)	90, 90, 90	90, 90, 120
Resolution range (Å)	60–2.60 (2.64–2.60)	54.72–1.95 (2.06–1.95)
Total No. of reflections	5650810	384625
No. of unique reflections	76823	20883
Completeness (%)	100 (100)	100 (100)
Multiplicity	14.3 (14.5)	18.4(17.7)
$\langle I/\sigma(I) \rangle$	15.1 (2.0)	19.6 (2.7)
<i>R</i> _{int} (%)	5.5 (41.7)	2.0 (30.8)
CC _{1/2} (%)	99.6 (85.2)	99.9 (84.7)
Overall <i>B</i> factor from Wilson plot (Å ²)	51.4	43.0

were cultured in LB medium containing 100 µg ml⁻¹ ampicillin at 310 K and 200 rev min⁻¹ until the OD₆₀₀ reached 0.5. The temperature was then decreased to 289 K before induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 20 h, the bacteria were harvested by centrifugation for 10 min at 6000g and 277 K.

After resuspension of the pellet in lysis buffer consisting of 25 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole, the cells were lysed in a nano homogenizer (ATS Engineering Inc., Canada) at 80 MPa pressure and 277 K. Insoluble debris was removed by centrifugation for 1 h at 12 000g and 277 K before applying the supernatant onto a pre-equilibrated HisTrap 5 ml column (GE Healthcare, Sweden). After washing with the same buffer containing 40 mM imidazole, the HpaA protein was eluted with an increasing imidazole concentration to 250 mM. After sample dilution 20-fold in a buffer consisting of 25 mM Tris pH 8.5, 25 mM NaCl, the pooled eluent was then loaded onto a HiTrap Q column (GE Healthcare, Sweden) using a linear gradient elution profile with an NaCl concentration from 25 to 1000 mM. A HiLoad 16/600 Superdex 75 column (GE Healthcare, Sweden) was used in the final step to polish the purification and buffer-exchange to 150 mM NaCl, 20 mM Tris pH 8.0. The purified protein was concentrated to 20 mg ml⁻¹ (measured using a BCA kit) before freezing in

aliquots at 193 K until use. The purity of the recombinant proteins was estimated by SDS-PAGE and Coomassie staining.

2.2. Crystallization

Initial screenings were carried out with a Gryphon-LCP robot (Art Robbins Instruments, USA) using the sitting-drop vapour-diffusion method with the Index, Crystal Screen, PEGRx, PEG/Ion and SaltRx screening kits from Hampton Research, California, USA. Drops were set up by mixing 0.4 µl protein solution and 0.4 µl reservoir solution and were equilibrated against 50 µl reservoir solution in 96-well sitting-drop plates (Bioray Instruments, People's Republic of China). The subsequent optimizations were manually conducted using either the hanging-drop or sitting-drop method with drops consisting of 1 µl protein solution and 1 µl reservoir reagent. All plates were incubated in a soundproof room at a constant temperature of 296 K.

Proteins produced from both constructs were crystallized and the optimal conditions for the growth of crystals used in X-ray data collection are provided in Table 2.

2.3. Data collection and processing

For data collection, the crystals grown in the lithium sulfate condition were directly mounted in a cryoloop and flash-cooled in liquid nitrogen because lithium sulfate can be used as an effective cryoprotectant (Rubinson *et al.*, 2000), while the crystals obtained from the ammonium sulfate condition were soaked in a cryoprotectant constituted of the mother liquor supplemented with 23%(w/v) D-(+)-glucose for several seconds before mounting and cooling. Diffraction data were collected at 100 K on beamline BL17U1 (Wang *et al.*, 2015) or BL18U1 at Shanghai Synchrotron Radiation Facility (SSRF), People's Republic of China. The collected data were indexed, integrated and scaled using *HKL-3000* (Minor *et al.*, 2006), *iMosflm* (Battye *et al.*, 2011) or *SCALA* from the *CCP4* program suite (Winn *et al.*, 2011). The statistics of data collection and processing are summarized in Table 3.

3. Results and discussion

The recombinant HpaA protein encompassing residues 31–260 (HpaA₃₁) was produced with high yield (approximately 28 mg purified protein from 1 l bacterial culture) and good purity (above 95% purity; lane 1 in Fig. 1a). The elution profile

from size-exclusion chromatography clearly revealed the monomeric form of the protein (Fig. 1*b*).

Three conditions were found for HpaA₃₁ in commercial screening kits (Hampton Research, USA). Cuboid-shaped crystals were obtained with good reproducibility from an optimal condition consisting of 1.8 M NaH₂PO₄/K₂HPO₄ pH 7.0 (Fig. 2*a*). These crystals, despite having a perfect appearance and a large size (100 × 100 × 70 µm), showed very poor diffraction capability (worse than 10 Å; Fig. 3*a*) on beamline BL17U1 (Wang *et al.*, 2015) or BL19U1 of SSRF. Unfortunately, further optimizations and post-crystallization treatments such as additive screening, crystal dehydration (Heras & Martin, 2005) and cryoprotectant screening all failed to improve the diffraction quality.

The poor diffraction of the HpaA₃₁ crystals prompted us to consider that intrinsically disordered regions may exist within the protein sequence which severely affect the packing in the crystals. Secondary-structure prediction using *Jpred4* (Drozdetskiy *et al.*, 2015) suggested that a long loop from residues 29 to 56 was probably unstructured (Supplementary Fig. S1). We thus made another construct by removing this potentially disordered loop in order to improve the quality of the crystal. The recombinant protein from this shorter construct comprising residues 53–260 (HpaA₅₃) was produced with a comparable yield and purity to HpaA₃₁ (lane 2 in Fig. 1*a*). Although purified HpaA₅₃ seemed to contain a trace amount of impurities that was barely visible on a SDS gel (the band between 15 and 25 kDa in lane 2; Fig. 1*c*), its crystallization was almost unaffected.

Large clustered crystals of HpaA₅₃ were observed in a condition consisting of 0.1 M HEPES pH 7.5, 1.5 M lithium sulfate two months after crystal screening was set up. A crystal with maximum dimensions of 400 × 70 × 30 µm (Fig. 2*b*) was

exposed to X-rays on beamline BL18U1, SSRF after manually removing the ‘branches’ before crystal mounting. This seemingly imperfect crystal diffracted to 2.5 Å resolution, with the collected data being processed in the hexagonal space group *P*6₅. The data quality, however, was not satisfactory for structure determination owing to a low mean *I*/σ(*I*) and a high Wilson *B* factor, which led us to consider optimization to further improve diffraction quality. The condition was refined using the hanging-drop method with grid screening of lithium sulfate concentration from 1.0 to 2.4 M and the pH value from 7.0 to 8.0. Unfortunately, the reproducibility of crystal growth under this condition was unexpectedly poor as only clear drops were observed after two months of equilibration. We speculated that nucleation might be a major obstacle to crystal growth in this case, and therefore sitting drops as well as microseeding were used to promote nucleation.

During the waiting time for crystal growth in the lithium sulfate condition, we tried to replace lithium sulfate with other sulfates such as magnesium sulfate and ammonium sulfate or with lithium salts such as lithium acetate and lithium chloride with the hope of reproducing isomorphous crystals, and found that HpaA₅₃ only crystallized with ammonium sulfate. Compared with the lithium sulfate condition, crystal growth under this condition showed a much faster growth rate and good reproducibility, but smaller sizes. In the optimal condition consisting of 0.1 M HEPES pH 8.0, 1.9 M ammonium sulfate, 5% (v/v) glycerol, rhomboid crystals with dimensions of 70 × 70 × 30 µm (Fig. 2*c*) were harvested within 7 d. A crystal grown using this condition diffracted to 2.6 Å resolution on beamline BL17U1 at SSRF (Fig. 3*b*), with a total of 360 images recorded. Data evaluation revealed that the crystal belonged to the orthorhombic space group *P*2₁2₁2, with unit-cell parameters *a* = 121.01, *b* = 190.56, *c* = 106.31 Å (Table 3).

Figure 1
(*a*) Analysis of purified HpaA₃₁ and HpaA₅₃ on a 17% SDS–PAGE gel. Lanes 1 and 2, purified HpaA₃₁ and HpaA₅₃ with theoretical molecular weights of 27.0 and 24.5 kDa, respectively; lane *M*, molecular-mass markers (labelled in kDa). (*b*) The elution profile of HpaA₃₁ from a Superdex 75 16/600 column. (*c*) HpaA₅₃ crystal content analysis on a 12% SDS–PAGE gel. Lane 1, dissolved HpaA₅₃ crystals obtained using lithium sulfate as precipitant; lane 2, purified HpaA₅₃; lane 3, dissolved HpaA₅₃ crystals obtained using ammonium sulfate as precipitant.

Eight HpaA monomers probably reside in the asymmetric unit, with a Matthews coefficient (V_M ; Matthews, 1968) of $3.13 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 60.66%.

In parallel, crystal growth using the lithium sulfate condition was successfully reproduced when the sitting-drop method was applied. Although they still grew slowly, crystals grew to larger sizes and, more importantly, were less clustered. After several rounds of optimization, the crystals obtained with 0.1 M HEPES pH 7.0, 1.92 M lithium sulfate could reach a maximum size of $800 \times 280 \times 60 \text{ \mu m}$ (Fig. 2*d*). One such crystal diffracted to 1.95 Å resolution on beamline BL18U1 at SSRF (Fig. 3*c*). The data set processed from 360 frames was indexed in space group $P6_5$, with unit-cell parameters $a = b = 95.42$, $c = 54.72 \text{ \AA}$, $\gamma = 120^\circ$. A V_M value (Matthews, 1968) of $2.94 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 58.11% were estimated, indicating the presence of only one HpaA molecule in the asymmetric unit. The statistics of the collected data showed a mean $I/\sigma(I)$ above 19 and a lower Wilson B factor (Table 3), indicating excellent quality of the crystal used for data collection.

The crystals obtained using both lithium sulfate and ammonium sulfate as the precipitant were confirmed to be authentic HpaA₅₃ crystals by SDS-PAGE (Fig. 1*c*). The structures were determined by molecular replacement using the crystal structure of an uncharacterized *H. pylori* protein (PDB entry 3bgh; New York SGX Research Center for Structural Genomics, unpublished work) as a search model.

In this study, we initially obtained large single crystals of HpaA₃₁ with a good appearance, which however showed very poor diffraction quality. Conversely, clustered crystals of HpaA₅₃ with irregular shapes diffracted to 1.95 Å resolution. This sharp contrast may serve as a good example indicating the lack of correlation between crystal appearance and interior packing, and suggests that the removal of a highly disordered sequence usually benefits crystal packing but may have little effect on the crystal shape. More interestingly, two crystal forms of HpaA₅₃ were obtained under similar conditions, both with sulfate as the precipitant but differing only in the cation composition. Empirically, small monovalent cations such as lithium and ammonium are thought to play similar

Figure 2 Crystals of HpaA₃₁ and HpaA₅₃. (a) Crystals of HpaA₃₁ grown under the optimal condition (1.8 M NaH₂PO₄/K₂HPO₄ pH 7.0) using the hanging-drop method. (b) Crystals of HpaA₅₃ grown in initial screening under the condition 0.1 M HEPES pH 7.5, 1.5 M lithium sulfate using the sitting-drop method. (c) Crystals of HpaA₅₃ grown under the optimal condition consisting of 0.1 M HEPES pH 8.0, 1.9 M ammonium sulfate, 5% (v/v) glycerol using the hanging-drop method. (d) Crystals of HpaA₅₃ grown under the optimized condition consisting of 0.1 M HEPES pH 7.0, 1.92 M lithium sulfate using the sitting-drop method.

roles in protein crystallization (McPherson, 2004), and thus isomorphous crystals would usually be expected to grow when lithium sulfate is replaced by ammonium sulfate or *vice versa*. In this case, however, crystals belonging to different space groups were grown, which seem to be solely determined by the presence of either lithium or ammonium ions in the mother liquor for crystallization. Apparently, the packing of HpaA molecules is differentially influenced by these cations. We

hence surmise that some cations may bind at the interface between protein monomers, although they cannot be distinguished from solvent molecules by electron density. Our experience described here seems to suggest that it may be worthwhile for crystallographers to consider changing monovalent cations in protein crystallization trials even if they have similar chemical properties.

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(a)

(b)

(c)

Figure 3

(a) Representative diffraction pattern of an HpaA₃₁ crystal using a PILATUS 3M detector on beamline BL19U1 at SSRF. (b) Representative diffraction pattern of an HpaA₅₃ crystal obtained in 0.1 M HEPES pH 8.0, 1.9 M ammonium sulfate, 5%(v/v) glycerol using an ADSC Q315R detector on beamline BL17U1 at SSRF. (c) Representative diffraction pattern of an HpaA₅₃ crystal grown using 0.1 M HEPES pH 7.0, 1.92 M lithium sulfate using a PILATUS3 6M detector on beamline BL18U1 at SSRF.

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