Recombinant expression, purification, crystallization and preliminary X-ray diffraction analysis of *Haemophilus influenzae* BamD and BamCD complex

Jintang Lei,^a Xun Cai,^a Xiaodan Ma,^a Li Zhang,^a Yuwen Li,^a Xue Dong,^a Joseph St Geme III^b and Guoyu Meng^{a*}

Received 21 October 2014

Accepted 15 December 2014

Keywords: *Haemophilus influenzae*; BamD; BamCD complex.

^aState Key Laboratory of Medical Genomics, Shanghai Institute of Hematology, Rui-Jin Hospital affiliated to Shanghai JiaoTong University School of Medicine, 197 Ruijin Er Road, Shanghai 200025, People's Republic of China, and ^bDepartment of Pediatrics, Children's Hospital of Philadelphia and the Perelman School of Medicine at the University of Pennsylvania, Philadelphia, USA. *Correspondence e-mail: guoyumeng@shsmu.edu.cn

The Bam machinery, which is highly conserved from bacteria to humans, is well recognized as the apparatus responsible for the insertion and folding of most outer membrane proteins in Gram-negative bacteria. In *Escherichia coli*, the Bam machinery consists of five components (*i.e.* BamA, BamB, BamC, BamD and BamE). In comparison, there are only four partners in *Haemophilus influenzae*: a BamB homologue is not found in its genome. In this study, the recombinant expression, purification, crystallization and preliminary X-ray diffraction analysis of *H. influenzae* BamD and BamCD complex are reported. The genes encoding BamC and BamD were cloned into a pET vector and expressed in *E. coli*. Affinity, ion-exchange and gel-filtration chromatography were used to obtain high-purity protein for further crystallographic characterization. Using the hanging-drop vapour-diffusion technique, BamD and BamCD protein crystals of suitable size were obtained using protein concentrations of 70 and 50 mg ml⁻¹, respectively. Preliminary X-ray diffraction analysis showed that the BamD crystals diffracted to 4.0 Å resolution and belonged to space group *P*₂₁₂₁₂₁, with unit-cell parameters *a* = 54.5, *b* = 130.5, *c* = 154.7 Å. The BamCD crystals diffracted to 3.8 Å resolution and belonged to space group *I*₂₁₂₁₂₁, with unit-cell parameters *a* = 101.6, *b* = 114.1, *c* = 234.9 Å.

1. Introduction

It has been suggested that 2–3% of the genes in Gram-negative bacterial genomes encode β -barrel outer membrane proteins (OMPs; Wimley, 2003). Recently, the molecular pathways that target OMPs to the outer membrane and that ensure their correct folding and insertion into the lipid bilayers have started to be unravelled (Voulhoux *et al.*, 2003; Wu *et al.*, 2005; Kim *et al.*, 2007). A highly conserved bacterial outer membrane protein known as Omp85 has been proposed to play an essential role in assisting the membrane insertion and folding of OMPs (Voulhoux *et al.*, 2003). Subsequently, Wu and coworkers discovered that this protein, known as YaeT in *Escherichia coli*, can form a functional heteromeric OMP complex with several lipoproteins (Wu *et al.*, 2005). Since then, the Omp85/YaeT complex has been recognized as an assembly platform for β -barrel OMPs (Gentle *et al.*, 2005; Knowles *et al.*, 2009; Ricci & Silhavy, 2012). Very recently, this complex was renamed the β -barrel assembly machinery (Bam). Its components, the outer membrane core Omp85/YaeT and its lipoprotein accessories YfgL, NlpB, YfiO and

SmpA, were renamed BamA, BamB, BamC, BamD and BamE, respectively (Knowles *et al.*, 2009; Ricci & Silhavy, 2012).

Haemophilus influenzae, a Gram-negative coccobacillus that causes at least three million clinical cases of disease worldwide each year, also shares the Bam machinery for OMP biogenesis. The BamA protein, known as *H. influenzae* D15, is proposed to help OMPs to insert and fold into the outer membrane (Thomas *et al.*, 2001; Meng *et al.*, 2006). Interestingly, sequence alignment using the *BLAST* search engine from the ExPASy server (<http://www.expasy.org/>) failed to identify a BamB homologue in the *H. influenzae* genome, suggesting that a different mode of assembly/architecture exists for the *H. influenzae* Bam machinery (Fig. 1). In *E. coli*, BamA and BamD are thought to form the core of the overall architecture, and both are essential for the biogenesis of outer membrane proteins (Wu *et al.*, 2005; Malinverni *et al.*, 2006). BamD can make a direct interaction with BamA (via its periplasmic POTRA domain), BamC and BamE (Malinverni *et al.*, 2006; Sklar *et al.*, 2007; Knowles *et al.*, 2011). The intermolecular interaction mediated by BamD is thought to help to sustain the functional and structural integrity of the whole Bam machinery (Malinverni *et al.*, 2006; Sklar *et al.*, 2007; Hagan *et al.*, 2010). In recent years, structural biology and functional studies have made significant contributions towards the understanding of the *E. coli* Bam machinery (Noinaj *et al.*, 2013; Gruss *et al.*, 2013; Knowles *et al.*, 2011; Sandoval *et al.*, 2011; Kim *et al.*, 2007, 2011; Gatzeva-Topalova *et al.*, 2008). However, the nature/mechanism of the intermolecular interactions among the components that lead to the overall assembly remains elusive. In their report of the BamCD structure, Kim and coworkers have shown that *E. coli* BamD can interact with the N-terminus of unstructured *E. coli*

BamC (Kim *et al.*, 2011). However, it is not clear whether the same mode of interaction is possible in *H. influenzae*, in which BamB is absent. In order to find out what roles BamD might play in the *H. influenzae* Bam machinery, we started a structural investigation of *H. influenzae* BamD and its interaction with BamC. Here, we report the recombinant expression, protein purification and crystallization of *H. influenzae* BamD and BamCD, which yielded crystals that diffracted to 4.0 and 3.8 Å resolution, respectively. This provides an important foundation for further crystallographic characterization and functional studies that might shed more light on the Bam lipoprotein accessories.

2. Materials and methods

2.1. Cloning

The PCR fragments encoding *H. influenzae* BamC and BamD were obtained using genomic DNA as a template. A BamD fragment, residues Ala29–Lys262, was inserted into pET-15b (Novagen) using the NdeI and BamHI sites. The recombinant BamD protein contained an N-terminal His tag and a thrombin cleavage site. The theoretical molecular weight of BamD is ~26.6 kDa. The BamC fragment, residues Ala45–Gln215, was cloned with a similar strategy using pET-32a (Novagen) and the BamHI and XhoI sites. The theoretical molecular weight of BamC is ~19.4 kDa. The restriction enzymes NdeI, XhoI and BamHI were obtained from New England Biolabs (NEB). The DNA polymerase KOD-Plus-, 2 mM dNTP, 25 mM MgSO₄ and 10× PCR buffer were purchased from Toyobo. The primers were designed as follows: for BamC, forward 5'-CGGGATCCTCGTCTAATCCTGAAACC-3' and reverse 5'-CCGCTCGAGTTATTGAATTAAAGTGCTTG-3', and for BamD, forward 5'-GGAATTCCATATGGCATCGGTAAATGAATTATACAC-3' and reverse 5'-CGGGATCCCTATTTTACTGCTGGCACTTTTAAAG-3'. PCR products were detected by electrophoresis on a 1.0% (w/v) agarose gel. The DNAs were purified using a gel-extraction kit (Qiagen). The digested PCR products were ligated into the expression vectors using T4 ligase (Takara), followed by transformation into *E. coli* BL21 (DE3) cells for protein expression.

2.2. Expression and purification

A fresh colony was used to inoculate Luria–Bertani (LB) broth supplemented with 50 mg l⁻¹ ampicillin. The bacterial culture was grown at 310 K for >12 h. A 10 ml overnight culture was transferred into 1 l LB medium (50 mg l⁻¹ ampicillin) for further incubation. For BamD, a cooling procedure was applied before induction. In brief, the cells were grown at 310 K until the OD₆₀₀ reached 0.9–1.0. The flasks containing the bacterial culture were immersed into ice water until the temperature of the cell culture decreased to 287 K. 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression. The cells were further incubated at 287 K for 18 h. For BamC, the bacterial culture was grown at 310 K until the OD₆₀₀ reached 0.5–0.6. 1 mM IPTG was then

Figure 1

The *H. influenzae* Bam machinery. BamC and BamD, which are crystallographically characterized in this report, are highlighted in underlined bold italic font. *H. influenzae* BamD is thought to interact directly with BamA, BamC and BamE. *H. influenzae* BamA consists of a β-barrel transmembrane domain and a periplasmic domain harbouring four POTRA subdomains labelled P1, P2, P3 and P4. Notably, BamB is absent in the *H. influenzae* genome.

added and the cells were further incubated for protein expression at 310 K for 4 h.

The cells were harvested by centrifugation at 4600g for 20 min. The pellet was resuspended in a buffer consisting of 20 mM Tris–HCl pH 8.0, 100 mM NaCl. The cells were disrupted with a French press. The cell debris was removed by centrifugation at 76 000g for 45 min. The clear lysate was loaded onto a 1 ml HisHP column (GE Healthcare). The column containing the BamD protein was washed with >50 column volumes of wash buffer consisting of 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 25 mM imidazole, followed by elution with a linear gradient of imidazole (0–300 mM) over 30 column volumes. For BamC, a similar purification strategy was used. The wash buffer consisted of 20 mM Tris–HCl pH 8.0, 100 mM NaCl. The elution buffer consisted of 20 mM Tris–HCl pH 8.0, 500 mM NaCl, 300 mM imidazole.

For both BamC and BamD, the recombinant protein after affinity chromatography was subjected to further thrombin (Sigma) digestion before it was dialyzed against a buffer consisting of 20 mM Tris–HCl pH 8.0, 150 mM NaCl for at least 4 h at 277 K. 50 U thrombin was incubated with ~10 mg His-BamC (or with 10 mg His-BamD) for >12 h. The cleaved His tag was removed on a 1 ml HisHP column (GE Healthcare) that was pre-equilibrated with buffer consisting of 20 mM Tris–HCl pH 8.0, 100 mM NaCl.

For BamD, the flowthrough fraction from the HisHP column was dialyzed against a buffer consisting of 20 mM MES pH 5.0, 20 mM NaCl before it was loaded onto a 1 ml SP column (GE Healthcare) pre-equilibrated with the same buffer. The column was then subjected to a wash procedure using a buffer consisting of 20 mM MES pH 5.0, 180 mM NaCl. A linear gradient of NaCl (180–600 mM, pH 5.0) was used for elution. Each protein sample was pooled and concentrated separately.

For BamC (*i.e.* the cleaved product after the HisHP column) and BamD, a final gel-filtration step with Sephacryl S100 (GE Healthcare) was used. Each protein sample was pooled separately and concentrated before it was loaded onto the S100 column pre-equilibrated with a buffer consisting of 20 mM Tris–HCl pH 8.0, 100 mM NaCl. The absorption at 280 nm was used to quantify the final protein concentration. SDS–PAGE was used to monitor the final purities of BamC and BamD (Fig. 2).

2.3. Crystallization of BamD

For initial crystallization trials, 240 crystallization conditions from JBScreen Classic 1–10 (Jena Bioscience) were used. The purified BamD protein was concentrated to 25 mg ml^{−1} using an Amicon device with a 10 kDa cutoff (Millipore). A standard hanging-drop vapour-diffusion setup using a 96-well plate from Hampton Research was used for protein crystallization. All crystallization screens were set up manually. Each droplet consisted of 0.3 µl protein sample and 0.3 µl crystallization reagent. The reservoir volume was 100 µl. The initial crystallization temperature was set to 293 K. After one week, small crystals of prism-like morphology could be observed in a

drop equilibrated against 15%(w/v) PEG 4000, 200 mM ammonium sulfate from JBScreen Classic 3.

For optimization, we set up further crystallizations at 277, 289 and 293 K. In parallel, a pH grid screen (pH 3.0–10.0) with 15%(w/v) PEG 4000, 200 mM ammonium sulfate was set up. Furthermore, the protein concentration was increased to >70 mg ml^{−1} for optimization. The final crystallization mixture consisted of 0.8 µl BamD protein (>70 mg ml^{−1}) and 0.8 µl reservoir solution [sodium acetate pH 5.0, 3%(w/v) PEG 4000, 100 mM ammonium sulfate]. The reservoir volume was 100 µl. The prism-like crystals grew to a size of 400 × 30 × 30 µm at 289 K (Fig. 3).

2.4. Crystallization of the BamCD complex

The purified BamC and BamD were mixed in a 1:1 molar ratio before they were subjected to a further gel-filtration purification step using Sephacryl S100 (GE Healthcare). The final protein complex was concentrated to 50 mg ml^{−1}. JBScreen Classic 1–10 (Jena Bioscience) was used for the initial trials. After one week of incubation at 289 K, cube-shaped crystals were observed among clouds of amorphous precipitate in a crystallization condition consisting of 10%(w/v) PEG 4000, 100 mM MES pH 6.5, 200 mM MgCl₂ from JBScreen Classic 2. After optimization, the final

Figure 2
Recombinant expression and purification of *H. influenzae* BamD and BamCD complex. (a) SDS–PAGE of BamD. Lane 1 contains a standard protein marker (labelled in kDa). Lanes 2 and 3 contain 2.5 and 25 µg purified BamD, respectively. (b) SDS–PAGE of BamCD. Lane 1 contains a standard protein marker (labelled in kDa). Lanes 2 and 3 contain 3.0 and 30 µg purified BamCD, respectively. (c) Gel-filtration chromatograms of purified BamD and BamCD.

crystallization condition was 3%(w/v) PEG 8000, 200 mM MgCl_2 , 100 mM MES pH 6.37 at 289 K. The crystallization mixture contained 0.8 μl protein and 0.8 μl crystallization reagent. The reservoir volume was 12 μl .

2.5. Crystallization of selenomethionine-derivatized BamCD complex

In order to solve the phase problem, purified selenomethionine-derivatized (SeMet) BamD was mixed with BamC before crystallization. To produce SeMet BamD, *E. coli* B834 (DE3) auxotroph cells were grown in methionine-deficient medium. Selenomethionine was added together with IPTG at induction. The SeMet BamD was purified using the same purification procedures as described above. SeMet BamCD complex crystals were obtained using crystallization conditions consisting of 3%(w/v) PEG 8000, 200 mM MgCl_2 , 100 mM MES pH 6.37.

(a)

(b)

Figure 3

Crystallization of *H. influenzae* BamD and BamCD. (a) BamD crystals were obtained using sodium acetate pH 5.0, 3%(w/v) PEG 4000, 100 mM ammonium sulfate and diffracted to 4 Å resolution. (b) BamCD crystals were obtained using 3%(w/v) PEG 8000, 200 mM MgCl_2 , 100 mM MES pH 6.37 and diffracted to 3.8 Å resolution.

2.6. Data collection

The crystals of BamD and of the BamCD complex were cryoprotected using 20%(w/v) PEG 400 in the presence of the final optimized crystallization condition before they were cooled to 100 K in liquid nitrogen. Diffraction data were collected on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF), People's Republic of China. The diffraction data were processed, integrated and scaled using *MOSFLM* and *SCALA* from *CCP4* (Winn *et al.*, 2011).

3. Results and discussion

Based on the sequence alignment, it appears that all of the members of the Bam machinery except for BamB are present in *H. influenzae*. Although this machinery is critical for OMP biogenesis, structural information concerning intermolecular interaction among lipoproteins is relatively sparse (Kim *et al.*, 2011). In this study, *H. influenzae* BamC and BamD could be expressed in *E. coli* with a high yield (>50 mg per litre of LB medium). The recombinant proteins were purified to >95% homogeneity via a series of chromatographic steps involving affinity, ion-exchange and gel-filtration columns (Fig. 2).

H. influenzae BamD, which can be stored at 277 K for several weeks without suffering protein degradation, was concentrated to 25 mg ml^{-1} before it was subjected to crystallization. Initial crystallization screening showed that 15%(w/v) PEG 4000 could be used to grow BamD crystals. In order to obtain crystals of sufficient size for X-ray diffraction, we employed two different strategies: (i) increasing the protein concentration to >70 mg ml^{-1} and (ii) lowering the precipitant concentration to <3%(w/v) PEG 4000. As shown in Fig. 3, a prism-like crystal of $400 \times 30 \times 30 \mu\text{m}$ was obtained. The BamD crystals were cryoprotected using 20%(w/v) PEG 400 in a stepwise fashion [45 s soaking time for each 5%(w/v) increment of PEG 400 in the optimized mother liquor]. A data set covering 360° of rotation was collected (0.6 s exposure, 1° oscillation, wavelength of 0.9792 Å) at SSRF. Initial processing and scaling of the data set suggested that the BamD crystal might belong to space group $P2_12_12_1$, with unit-cell parameters $a = 54.5$, $b = 130.5$, $c = 154.7$ Å and four molecules in the asymmetric unit.

In order to crystallize the BamCD complex, purified BamC was mixed with BamD in a 1:1 molar ratio and further incubated at 289 K for more than 12 h. The BamCD complex was subjected to further purification by gel-filtration chromatography (Fig. 2). As initial trials, JBScreen Classic 1–10 containing 240 conditions was set up at 277, 289 and 293 K. A promising hit from JBScreen Classic 2 containing PEG 4000 as the major precipitant was observed at 289 K. Further grid screens were set up to determine the best percentage of PEG 4000 to use. However, no crystals that were sufficiently large could be obtained using this approach. A revised optimization strategy was then employed in which the precipitant PEG 4000 was replaced by PEG 400, PEG 550, PEG 1000, PEG 2000, PEG 3000, PEG 4000, PEG 6000, PEG 8000, PEG 10 000 or PEG 20 000. The best crystallization condition was observed

to be 3%(w/v) PEG 8000, 200 mM MgCl₂, 100 mM MES pH 6.37 at 289 K, in which the BamCD crystal could achieve dimensions of 50 × 50 × 50 µm (Fig. 3). For data collection, the BamCD crystals were cryoprotected in the same stepwise fashion as the BamD crystals using 20%(w/v) PEG 400 in the presence of 3%(w/v) PEG 8000, 200 mM MgCl₂, 100 mM MES pH 6.37. The BamCD crystals diffracted to 3.8 Å resolution. Initial processing and scaling of the data set suggested that the BamCD crystal might belong to space group *I*2₁²₁2₁, with unit-cell parameters *a* = 101.6, *b* = 114.1, *c* = 234.9 Å.

Acknowledgements

We thank the personnel of beamline BL17U at SSRF, Shanghai, People's Republic of China for help during data collection. This work was supported by a research grant (81370620) from NNSFC (to GM), a research grant (11JC1407200) from SMSTC (to GM), a research grant (12ZZ109) from SME (to GM), the Program for New Century Excellent Talents in University (NCET-10-9571; to GM) and The Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning (to GM).

References

- Gatzeva-Topalova, P. Z., Walton, T. A. & Sousa, M. C. (2008). *Structure*, **16**, 1873–1881.
- Gentle, I. E., Burri, L. & Lithgow, T. (2005). *Mol. Microbiol.* **58**, 1216–1225.
- Gruss, F., Zähringer, F., Jakob, R. P., Burmann, B. M., Hiller, S. & Maier, T. (2013). *Nature Struct. Mol. Biol.* **20**, 1318–1320.
- Hagan, C. L., Kim, S. & Kahne, D. (2010). *Science*, **328**, 890–892.
- Kim, K. H., Aulakh, S. & Paetzel, M. (2011). *J. Biol. Chem.* **286**, 39116–39121.
- Kim, S., Malinverni, J. C., Sliz, P., Silhavy, T. J., Harrison, S. C. & Kahne, D. (2007). *Science*, **317**, 961–964.
- Knowles, T. J. *et al.* (2011). *EMBO Rep.* **12**, 123–128.
- Knowles, T. J., Scott-Tucker, A., Overduin, M. & Henderson, I. R. (2009). *Nature Rev. Microbiol.* **7**, 206–214.
- Malinverni, J. C., Werner, J., Kim, S., Sklar, J. G., Kahne, D., Misra, R. & Silhavy, T. J. (2006). *Mol. Microbiol.* **61**, 151–164.
- Meng, G., Surana, N. K., St Geme, J. W. III & Waksman, G. (2006). *EMBO J.* **25**, 2297–2304.
- Noinaj, N., Kuszak, A. J., Gumbart, J. C., Lukacik, P., Chang, H., Easley, N. C., Lithgow, T. & Buchanan, S. K. (2013). *Nature (London)*, **501**, 385–390.
- Ricci, D. P. & Silhavy, T. J. (2012). *Biochim. Biophys. Acta*, **1818**, 1067–1084.
- Sandoval, C. M., Baker, S. L., Jansen, K., Metzner, S. I. & Sousa, M. C. (2011). *J. Mol. Biol.* **409**, 348–357.
- Sklar, J. G., Wu, T., Gronenberg, L. S., Malinverni, J. C., Kahne, D. & Silhavy, T. J. (2007). *Proc. Natl Acad. Sci. USA*, **104**, 6400–6405.
- Thomas, K. L., Leduc, I., Olsen, B., Thomas, C. E., Cameron, D. W. & Elkins, C. (2001). *Infect. Immun.* **69**, 4438–4446.
- Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M. & Tommassen, J. (2003). *Science*, **299**, 262–265.
- Wimley, W. C. (2003). *Curr. Opin. Struct. Biol.* **13**, 404–411.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Wu, T., Malinverni, J., Ruiz, N., Kim, S., Silhavy, T. J. & Kahne, D. (2005). *Cell*, **121**, 235–245.