Purification and preliminary X-ray analysis of YbeY from *Staphylococcus aureus*

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Endoribonuclease YbeY is essential for maturing ribosomal RNA in a wide range of bacteria. YbeY is specific to the single-stranded RNA of ribosomal RNAs and small RNAs. However, the structural relationship with the function and recognition of substrates remains elucidated. In this study, we overexpressed full-length YbeY from the gram-positive bacterium *Staphylococcus aureus*. The protein was purified and crystallized under conditions containing 0.1 M sodium citrate (pH 5.0) and 44% (w/v) polyethylene glycol 600. An X-ray diffraction dataset at a resolution of 1.9 Å was collected, and the dataset belonged to space group $P6_3$ with unit cell parameters $a = 100.7$ and $c = 50.2$ Å. The single-wavelength anomalous diffraction method was successful in determining the crystal structure. We are now refining the structure combined with structural analysis. The structure will provide molecular insights into the substrate recognition mechanism by showing the high-resolution features of the active sites.

INTRODUCTION

RNA molecules are synthesized by RNA polymerases using DNA as templates and processed or degraded by diverse ribonucleases (Raines, 1998). Regulation of mRNA stability by ribonucleases is essential for adapting bacteria to changing environments. YbeY is a highly conserved endoribonuclease specific to the single-stranded RNA of ribosomal RNAs (Davies and Walker, 2008; Davies et al., 2010; Liu et al., 2015). Early studies revealed that YbeY is involved in late-stage 70S ribosome quality control and maturation of the 3' terminus of the 16S rRNA in bacteria (Jacob et al., 2013; Vercruysse et al., 2014; Liu et al., 2015). YbeY also plays a vital role in the adaption process of cells by directly modifying mRNAs or processing small RNAs (sRNAs), which regulate mRNA translation and stability (Pandey et al., 2014; Saramago et al., 2017).

Transcriptomic analyses in the opportunistic bacteria *Pseudomonas aeruginosa* revealed that the deletion of *ybeY* gives rise to downregulation of catalase genes with increased susceptibility to oxidative stress (Xia et al., 2020a). A subsequent study further revealed the pleiotropic roles of YbeY and YbeY-mediated regulation in adapting to environmental stress, which was found to be associated with bacterial virulence (Xia et al., 2020b). In *P. aeruginosa*, *E. coli*, and *S. aureus*, the *ybe* operon contains the *ybeY* and *ybeZ* genes with ATP binding and a nucleoside triphosphate hydrolase domain (Butland et al., 2005; Vercruysse et al., 2016; Wood et al., 2019). However, it remains unclear how YbeY contributes to bacterial virulence and the stress response at the molecular level.

The crystal structure of YbeY from *Escherichia coli* was determined at a 2.7-Å resolution by a structural genomics

consortium group before the function of YbeY was known (Zhan et al., 2005). The structure showed that YbeY binds a Ni ion coordinated by three histidine residues and presumably one water molecule in a tetrahedral geometry. The Ni ion was from the purification step using Ni-NTA resin, and it was unknown if the Ni ion was required for the activity of YbeY. A later study revealed that Zn ions but not Ni ions are necessary for enzymatic activity (Babu et al., 2020). However, the Zn-bound and/or substrate-bound structure needs to be determined to investigate the action mechanism of the highly conserved YbeY. In this study, we report the crystallization and preliminary X-ray analysis of YbeY from *S. aureus*, which contained Zn ions and substrate surrogate molecules at the active site.

MATERIALS AND METHODS

Construction and protein expression

The gene which encodes the YbeY (NCBI reference sequence: BAB57732.1) protein was amplified using PCR from the genomic DNA of the *Staphylococcus aureus* strain Mu50. First, the products of PCR were cleaved by the restriction enzymes *NcoI* and *XhoI*. Then, the digested sticky-ended PCR products were inserted between the two enzyme sites in the pET28a (Merck, USA). The detailed cloning information is presented in Table 1. The resulting pET28a-SaYbeY vector expresses full-length YbeY with an uncleavable hexaHis tag for purification at the C-terminal region of YbeY.

Protein overexpression and purification

The pET28a-SaYbeY vector was transformed into the *E. coli* strain BL21 (DE3) to overexpress the YbeY protein. Then, the

BL21 cell was cultured in 1.5 L of LB medium with 100 µg/ml ampicillin at 37°C. When the OD₆₀₀ value reached 0.6, IPTG at a final concentration of 0.5 mM was added to the LB medium to induce the YbeY protein. The cells were further cultured at 30°C for 6 h after induction. Then, the cells were harvested by high-speed centrifugation (1,400×g) for 7 min at 4°C. Next, the cell pellet was resuspended in 50 ml of cell resuspension buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl and 2 mM β-mercaptoethanol. A sonicator disrupted the bacterial cell walls at 4°C, and the cell debris was removed by centrifugation at 20,000×g for 30 min at 4°C.

Ni-NTA agarose resin (1 ml; Qiagen, Germany) was mixed with 50 ml of the cell lysate solution and gently rolled in a column using a blood mixer roller for 45 min at 4°C. The loosely bound proteins were washed out with 250 ml of cell resuspension buffer supplemented with 20 mM imidazole (pH 8.0). Then, the protein was subsequently eluted with 30 ml of cell resuspension buffer supplemented with 250 mM imidazole (pH 8.0). The pooled fractions containing the YbeY protein were further purified by anion exchange chromatography. An anion exchange chromatographic column (Hitrap Q; GE Healthcare, USA) was applied in a gradient against cell resuspension buffer supplemented with 1 M NaCl. The protein was eluted in a range of 400–450 mM NaCl. The pooled fractions were further purified using a gel filtration chromatographic column (HiLoad™ 26/610 Superdex™ 200 pg; GE Healthcare, USA) in cell resuspension buffer. The resulting protein solution was concentrated to 11 mg/ml using a Vivaspin centrifugal concentrator (10 kDa molecular-weight cutoff; Millipore, USA), measured in Bradford solution (Pierce, USA) using BSA as the standard. The concentrated protein sample was stored frozen at -80°C until use.

Crystallization

Since YbeY requires Zn ions for activity, 1 mM ZnSO₄ was added to the protein sample (11 mg/ml) in a buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl and 2 mM β-mercaptoethanol before crystallization. A sitting-drop vapor-diffusion method was applied using the automated crystal screening device MOSQUITO

TABLE 1 | *S. aureus* YbeY production information

YbeY	
Source organism	<i>Staphylococcus aureus</i> Mu50
DNA source	Genomic DNA
Forward primer	GCGCCATGGCATTACGATAGATTTAGCG
Reverse primer	GCGCTCGAGGTCTCGTGTTAATCC
Expression vector	pET28a
Expression host	<i>E. coli</i> BL21 (DE3)
The complete amino acid sequence of the construct produced	MAFTIDFSDHTGLVKDAWYKQIEDLLEFA KKEEHIEDDAELSVTFVDKQEIQINRTYR DKDKVTDVISFALEEDEPEIDFSGLDIPRVL GDIIICTDVAQEQANNYGHGSFERELGFLAL HGFLHLLGYDHMTADEKEMFGRQDTIL NAYGLTRDHHHHH

(SPT Labtech, USA) for primary crystallization screening of the YbeY protein. The primary screened crystals with MCSG-2T Crystallization Suite (Anatrace, USA) were observed under one condition over 20 days. The crystals were found in a reservoir containing 0.1 M sodium citrate (pH 5.5) and 40% (w/v) polyethylene glycol 600 in 96-well plates stored at 14°C. The final data-collectible crystals were grown via the hanging-drop diffusion method under a reservoir solution containing 0.1 M sodium citrate (pH 5.0), 44% (w/v) polyethylene glycol 600, and 1 mM ZnSO₄ at 14°C in a 15-well plate.

Data collection and processing

The crystals were transferred to 2 µl of the viscous oil Paraton-N with a brief incubation for 1 s for cryoprotection. Then, the crystals were manually plunged into liquid nitrogen at -173°C for flash freezing. The 360°-coverage datasets were collected with 1° oscillation and 0.1-s exposure from a single crystal in the 5C beamline of the Pohang Accelerator Laboratory, the Republic of Korea, which was equipped with an Eiger 9M detector (DECTRIS, Switzerland). The single-wavelength anomalous diffraction (SAD) datasets were collected at a wavelength of 1.2822 Å. The diffraction datasets were processed, merged, and scaled with the HKL-2000 program suite (Otwinowski and Minor, 1997) (see Table 2 for the detailed data collection statistics). The structure of YbeY was determined using the single-wavelength anomalous diffraction (SAD) method with AutoSol in PHENIX (Terwilliger et al., 2009; Adams et al., 2010). The model building and refinement of the structure are now underway using COOT and PHNINX REFINE (Emsley and Cowtan, 2004; Afonine et al., 2010; Afonine

TABLE 2 | X-ray diffraction data

YbeY	
Data collection	
Beamline	PAL 5C
Wavelength (Å)	1.28176
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	0.1
Space group	P63
Cell dimensions	
a, c (Å)	100.7, 50.2
Resolution (Å)	50.0-1.90 (1.93-1.90)
Total No. reflections	22303 (1017)
R _{merge}	0.056 (0.184)
High-resolution shell CC _{1/2}	0.988
I/σI	56 (14.6)
Completeness (%)	96.6 (88.7)
Anomalous completeness (%)	96.1
Redundancy	21.1 (18.8)

*The values in parentheses are for the highest resolution shell.

et al., 2012).

RESULTS AND DISCUSSION

YbeY is an essential gene of *S. aureus* and might be a drug target to control pathogenic bacteria. The *ybeY* gene was amplified from the genomic DNA of *S. aureus* MU50 by PCR. We inserted the PCR product into the pLIC vector, which expresses full-length YbeY with the N-terminal hexaHis tag and the TEV protease recognition site for cleavage of the hexaHis tag. Unfortunately, we could not completely cleave the hexaHis tag with the recombinant TEV protease, although the purification of the proteins was successful at a high yield. Unfortunately, we failed to obtain crystals with purified proteins. Later, we moved the hexaHis tag to the C-terminal region using the *Nco*I and *Xho*I enzyme sites in the pET28a vector, resulting in pET28a-SaYbeY. Full-length YbeY from *S. aureus* was expressed in the *E. coli* expression system at 30°C after induction by 0.5 mM IPTG. The YbeY protein with the C-terminal hexaHis tag was purified using serial chromatography: Ni-NTA affinity, anion exchange, and size exclusion chromatography. Since the C-terminal hexaHis tag is not cleavable, the tag-removing step was not necessary. The protein band indicated that the YbeY protein has a molecular weight of 18 kDa on the SDS polyacrylamide gel, which is well matched to the theoretical value of the full-length YbeY protein with the hexaHis tag (18.7 kDa) (Figure 1). The final yield was 12 mg from a 1-liter culture, and the protein was concentrated to 11 mg/ml. The details of the cloning and expression of the protein

FIGURE 1 | YbeY protein band on an SDS-polyacrylamide gel. The left lane indicates the size markers of protein (kDa). The protein band indicates that YbeY with the C-terminal hexaHis tag has a molecular weight of 18 kDa on a 4–20% gradient SDS polyacrylamide gel (Bio-Rad).

are described in Table 1.

We added ZnSO_4 to the protein sample at a final concentration of 1 mM to sufficiently supply Zn ions to the YbeY protein. The primary screened crystals exhibited the shapes of piles of the sliced rods, indicating that the crystallization condition needed to be optimized. The optimization work was performed by adjusting the pH and concentration of polyethylene glycol 600 at 14°C in a 15-well plate using the hanging-drop diffusion method. ZnSO_4 was further added to the reservoir solution. The final data-collectible crystals were grown under a reservoir solution containing 0.1 M sodium citrate (pH 5.0), 44% (w/v) polyethylene glycol 600, and 1 mM ZnSO_4 (Figure 2).

To collect X-ray data of YbeY, we transferred crystals to viscous oil paratone-N for cryoprotection. Then, the crystal was flash-frozen by a manual plunge into liquid nitrogen. The X-ray diffraction data were collected on the beamline 5C at PLS at -173°C (Figure 3). All 360 frames were collected using a 1° oscillation angle at the wavelength of the Zn absorption K edge ($1.28176 \text{ \AA}$), which was adequate to collect anomalous signals from the bound Zn ion. The crystal data set was processed at a resolution of $1.9 \text{ \AA}$ and belonged to the primitive hexagonal space group with unit cell parameters of $a = 100.7$ and $c = 50.2 \text{ \AA}$. The program suggested spacegroup $P6_3$, and consistently, the systemic absence was found at diffractions of $h = 0, k = 0$, and $l = 2n + 1$. The cell content analysis with the Matthews coefficient by Xtriage in PHENIX was $4.08 \text{ \AA}^3 \text{ Da}^{-1}$ and suggested that one protomer of YbeY is present in an asymmetric unit.

We attempted single-wavelength anomalous diffraction (SAD) with Zn ions to determine the structure of YbeY, and the anomalous completeness was 96.1%. The statistics for the diffraction data are summarized in Table 2. The single-wavelength anomalous diffraction method was successful in

FIGURE 2 | A crystal of the *S. aureus* YbeY protein. The approximate dimensions of the crystal are $0.25 \times 0.1 \times 0.25 \text{ mm}$.

FIGURE 3 | X-ray diffraction image of the YbeY protein. Two circles with the dotted line show resolutions for 2.0 Å and 3.0 Å of the diffraction data. The blue rectangular site in the top left corner is enlarged in the bottom left corner.

determining the crystal structure using the program AutoSol in PHENIX (Terwilliger et al., 2009; Adams et al., 2010). The initial electron density map showed the presence of the Zn ion at the putative active site of one protomer in the asymmetric unit. We are refining the structure with the model building with COOT (Emsley and Cowtan, 2004). Our structure will provide molecular insights into the substrate recognition mechanism by showing the high-resolution features of the active sites. Furthermore, the structure will help develop drugs to control the pathogenic bacteria *S. aureus*.

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REFERENCES

Adams, P.D., Afonine, P.V., Bunkóczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., McCoy, A.J., Moriarty, N.W., Oeffner, R., Read, R.J., Richardson,

D.C., et al. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213–221.

Afonine, P.V., Grosse-Kunstleve, R.W., Echols, N., Headd, J.J., Moriarty, N.W., Mustyakimov, M., Terwilliger, T.C., Urzhumtsev, A., Zwart, P.H., and Adams, P.D. (2012). Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr D Biol Crystallogr* **68**, 352–367.

Afonine, P.V., Mustyakimov, M., Grosse-Kunstleve, R.W., Moriarty, N.W., Langan, P., and Adams, P.D. (2010). Joint X-ray and neutron refinement with phenix.refine. *Acta Crystallogr D Biol Crystallogr* **66**, 1153–1163.

Babu, V.M.P., Sankari, S., Budnick, J.A., Caswell, C.C., and Walker, G.C. (2020). Sinorhizobium meliloti YbeY is a zinc-dependent single-strand specific endoribonuclease that plays an important role in 16S ribosomal RNA processing. *Nucleic Acids Res* **48**, 332–348.

Butland, G., Peregrín-Alvarez, J.M., Li, J., Yang, W., Yang, X., Canadien, V., Starostine, A., Richards, D., Beattie, B., Krogan, N., Davey, M., Parkinson, J., Greenblatt, J., and Emili, A. (2005). Interaction network containing conserved and essential protein complexes in *Escherichia coli*. *Nature* **433**, 531–537.

Davies, B.W., Köhrer, C., Jacob, A.I., Simmons, L.A., Zhu, J., Aleman, L.M., Rajbhandary, U.L., and Walker, G.C. (2010). Role of *Escherichia coli* YbeY, a highly conserved protein, in rRNA processing. *Mol Microbiol* **78**, 506–518.

Davies, B.W., and Walker, G.C. (2008). A highly conserved protein of unknown function is required by *Sinorhizobium meliloti* for symbiosis and environmental stress protection. *J Bacteriol* **190**, 1118–1123.

Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **60**, 2126–2132.

Jacob, A.I., Köhrer, C., Davies, B.W., Rajbhandary, U.L., and Walker, G.C. (2013). Conserved bacterial RNase YbeY plays key roles in 70S ribosome quality control and 16S rRNA maturation. *Mol Cell* **49**, 427–438.

Liu, J., Zhou, W., Liu, G., Yang, C., Sun, Y., Wu, W., Cao, S., Wang, C., Hai, G., Wang, Z., Bock, R., Huang, J., and Cheng, Y. (2015). The conserved endoribonuclease YbeY is required for chloroplast ribosomal RNA processing in *Arabidopsis*. *Plant Physiol* **168**, 205–221.

Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* **276**, 307–326.

Pandey, S.P., Winkler, J.A., Li, H., Camacho, D.M., Collins, J.J., and Walker, G.C. (2014). Central role for RNase YbeY in Hfq-dependent and Hfq-independent small-RNA regulation in bacteria. *BMC Genomics* **15**, 121.

Raines, R.T. (1998). Ribonuclease A. *Chem Rev* **98**, 1045–1066.

Saramago, M., Peregrina, A., Robledo, M., Matos, R.G., Hilker, R., Serrania, J., Becker, A., Arraiano, C.M., and Jiménez-Zurdo, J.I. (2017). *Sinorhizobium meliloti* YbeY is an endoribonuclease with unprecedented catalytic features, acting as silencing enzyme in riboregulation. *Nucleic Acids Res* **45**, 1371–1391.

Terwilliger, T.C., Adams, P.D., Read, R.J., McCoy, A.J., Moriarty, N.W., Grosse-Kunstleve, R.W., Afonine, P.V., Zwart, P.H., and Hung, L.W. (2009). Decision-making in structure solution using Bayesian estimates of map quality: the PHENIX AutoSol wizard. *Acta Crystallogr D Biol Crystallogr* **65**, 582–601.

Vercruysse, M., Köhrer, C., Davies, B.W., Arnold, M.F., Mekalanos, J.J., Rajbhandary, U.L., and Walker, G.C. (2014). The highly conserved bacterial RNase YbeY is essential in *Vibrio cholerae*, playing a critical role in virulence, stress regulation, and RNA processing. *PLoS Pathog* **10**, e1004175.

Vercruysse, M., Köhrer, C., Shen, Y., Proulx, S., Ghosal, A., Davies, B.W., Rajbhandary, U.L., and Walker, G.C. (2016). Identification of YbeY-Protein

Interactions Involved in 16S rRNA Maturation and Stress Regulation in *Escherichia coli*. *mBio* **7**, e01785-16.

Wood, A., Irving, S.E., Bennison, D.J., and Corrigan, R.M. (2019). The (p)ppGpp-binding GTPase Era promotes rRNA processing and cold adaptation in *Staphylococcus aureus*. *PLoS Genet* **15**, e1008346.

Xia, Y., Weng, Y., Xu, C., Wang, D., Pan, X., Tian, Z., Xia, B., Li, H., Chen, R., Liu, C., Jin, Y., Bai, F., Cheng, Z., Kuipers, O.P., and Wu, W. (2020a). Endoribonuclease YbeY is essential for RNA processing and virulence in *Pseudomonas aeruginosa*. *mBio* **11**, e00659-20.

Xia, Y., Xu, C., Wang, D., Weng, Y., Jin, Y., Bai, F., Cheng, Z., Kuipers, O.P., and Wu, W. (2020b). YbeY controls the type III and type VI secretion systems and biofilm formation through RetS in *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **87**, e02171-20.

Zhan, C., Fedorov, E.V., Shi, W., Ramagopal, U.A., Thirumuruhan, R., Manjasetty, B.A., Almo, S.C., Fiser, A., Chance, M.R., and Fedorov, A.A. (2005). The ybeY protein from *Escherichia coli* is a metalloprotein. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **61**, 959-963.