# Purification and Preliminary Crystallographic Studies of CutC, a Novel Copper Homeostasis Protein from *Shigella flexneri*

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**Abstract:** CutC is a novel copper homeostasis protein containing 248 amino acids. Here we report the cloning, expression, purification, crystallization and preliminary X-ray crystallographic studies of CutC from *Shigella flexneri 2a*. Purification of CutC and its selenomethionine (SeMet) derivate were done using immobilized metal ion affinity chromatography, size-exclusion and ion-exchange chromatography. The purified proteins were crystallized using the hanging drop vapor diffusion method. The diffraction data for the native and SeMet CutC, respectively, have been collected with resolution of 1.7 Å and 2.1 Å. They belong to the space group C2221 and similar cell dimension. The native protein crystals have cell parameters: a=75.3267, b=97.6718, c=132.6910.

**Keywords:** CutC, Copper homeostasis protein, purification, crystallization, preliminary crystallographic studies, *Shigella flexneri*.

## 1. INTRODUCTION

Heavy metals play critical roles in biological metabolic processes. They can maintain cellular osmotic balance and function as cofactors of enzymes and as stabilizers of protein structures. However, excess heavy metals are very harmful to cells, even resulting in cell death. They can bind to essential cellular components to change their activities. Copper is a heavy metal trace element that is essential in all organisms and which plays a vital role in the growth and physiology of the cells [1]. Copper ions are involved in many redox reactions since it can exist in two states, either in an oxidized state as Cu(II) or in a reduced state as Cu(I) [2]. Proteins containing copper ions are widely distributed in aerobic organisms and are involved in important physiological processes such as respiration, iron transport, oxidative stress protection, blood clotting and pigmentation [3]. Although it is necessary for cellular metabolism, copper, like other heavy elements, is highly toxic when it is present in excess. Deficiency in copper proteins, or an alteration of their activities, often cause disease state or pathophysiological conditions such as the Menkes and Wilson diseases, the Alzheimer pathology, and the Creutzfeld-Jacob syndrome of human and *ctr1* mutants of *Saccharomyces cerevisiae* [1,4]. It is therefore critical for aerobic organisms to regulate the cellular copper level in time to avoid its toxicity. All organisms must have their own copper homeostasis mechanisms. These mechanisms are mainly carried out by proteins which specifically bind copper in the cell [4].

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The copper homeostasis mechanism in the bacteria is not clearly understood. There are two types of genes appear to be associated with the copper level regulation. One of these is the *cop* genes family which comprises four members: *cop*A, *cop*B, *cop*Y, *cop*Z [5]. This is a well-understood system of active transport efflux pumps. Another is the *cut* genes family that possesses six *cut* gene members: *cut*A, *cut*B, *cut*C, *cut*D, *cut*E, and *cut*F [1]. The functions of their products are not very clear. CutA, the product of *cut*A, is a relatively small protein of molecular mass about 12 kDa [6]. The *cut*E gene encodes apolipoprotein N-acyltransferase. CutE might function in the acylation of CutC and other apolipoproteins required for copper tolerance. CutF is an outer membrane lipoprotein, and apo-CutF is attached to fatty acids by CutE [1, 6, 7]. The roles of CutB and CutD are still not clear.

The product of *cut*C is a protein (CutC) containing 248 amino acid residues. CutC has a MPRMEDIM sequence, which is similar to the copper-binding motif MXXXXMXXM. The *cut*C deletion strain showed copper sensitivity at high copper concentrations [2]. Some studies have implicated that CutC plays a role in intracellular trafficking of Cu(I) [8,9]. According to these, CutC may be a cytoplasmic copper-binding protein.

To understand the detailed structure-function relationship, three dimensional structure of CutC would be significant. We have cloned the *cut*C gene, and expressed CutC as a C-terminally $His_6$-tagged protein in *E. coli*. The purified protein is used for crystallization. Here we report the crystallization and preliminary X-ray diffraction analysis of both native and SeMet CutC.

## 2. MATERIALS AND METHODS

### 2.1. Cloning of CutC from *Shigella flexneri*

Genomic DNA from *Shigella flexneri 2a Str. 301* is isolated using genomic DNA purification system kit

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(Promega).Two specific PCR Primers 5'TACTGGGC ATATGGCATTACT GGAAATTTGCTGTT 3'(sense) and 5'ATTCTCGAGTTT GGCCTGATGGCGTTC 3'(anti-sense) were designed from *cut*C gene sequence (GenBank: NP_707762) to amplify the full length gene from genomic DNA of *shigella flexneri 2a* including NdeI and XhoI restriction sites, respectively. The PCR product was purified using DNA purification kit (Promega). The purified DNA was inserted into pET22b(+) (Novagen) using the NdeI and XhoI restriction enzymes and T4 DNA ligase to generate the expression plasmid pET22b-CutC. The plasmid was amplified in TG1-competent *E. coli* cells. The construct was confirmed by sequencing.

### 2.2. Expression and Purification

The recombinant plasmid was transformed into *E. coli* host strain Bl21 codon plus competent cell. The transformed bacteria were grown on the LB plate containing ampicillin (100 μg $ml^{-1}$) and chloramphenicol (30 μg $ml^{-1}$) overnight at 37 ºC. The single clone was picked into 5ml LB medium and cultured overnight. 1.5 ml overnight culture was subculture into 50ml of fresh LB. 3 hours later, 30 ml culture from the 50 ml bacteria culture was transferred into 1 L fresh LB medium. All LB mediums used contain ampicillin (100 μg $ml^{-1}$) and chloramphenicol (30 μg $ml^{-1}$). The cells were induced with 1mM isopropyl β-D-thiogalactoside (IPTG) when the culture reached an $O.D_{600}$ of 0.6-0.8. After induction, the cultivation was continued for 3h. The cells were harvested at 4000 rpm at 4 ºC for 30 minutes. The bacteria obtained were saved at -20 ºC for later use.

The purification was completed with Ni-NTA affinity column (Novegan) and AKTA purification system(Amersham Pharmacia).The cell pellet was resuspended in 30 ml Lysis Buffer (50mM $NaH_2PO_4$, pH8.0; 300 mM NaCl; 10mM imidazole) with 0.1 mM PMSF and the cells were broken using FRENCH Pressure Cell in two cycles. The lysate was centrifuged at 12,500 rpm at 4 ºC for 20 min. The supernatant was loaded directly into Ni-NTA column (Novagen), which was pre-equilibrated with Lysis Buffer. The column was washed with Wash Buffer (50 mM $NaH_2PO_4$, pH8.0; 300 mM NaCl; 20 mM imidazole). CutC containing $His_6$-tag in its C-terminal was eluted by Elution Buffer (50 mM $NaH_2PO_4$, pH 8.0; 300 mM NaCl; 250 mM imidazole). The eluate was concentrated by ultra-filtration (Millipore) and loaded into Superdex75 HR16/60 column (Amersham Pharmacia) pre-equilibrated with buffer of 50 mM $NH_4HCO_3$ at 20 ºC. The column flow rate was 1ml/min and all peak fractions were collected. All fractions were identified be SDS-PAGE. The fraction containing CutC was concentrated by ultra-filtration again, and loaded into global Mono Q HR5/5 (Amersham Pharmacia) pre-equilibrated with buffer A (50 mM Tris-HCl, pH8.0). The column was washed with linear gradient of Buffer A and Buffer B (50 mM Tris-HCl, pH8.0, 1M NaCl) within 20 column volume. The CutC was eluted at the gradient of about 25% of buffer B. The fraction was collected and desalted with Hitrap Desalting column. The protein was concentrated for crystallization.

The method of inhibition to methionine metabolism pathway was used to get the selenomethionine substituted (SeMet) derivative. The transformed Bl21 codon plus cells were grown in minimal medium at 37 ºC. When $O.D_{600}$ of culture reached 0.6-0.8, solid amino acid supplements (Lys, Phe, Thr, Ile, Leu, Val, SeMet) were added to the culture. After 15 minutes, the expression was induced as usual [10, 11]. The purification of SeMet protein was performed using the same method as that for native protein.

### 2.3. Crystallization

The native and SeMet derivate protein were concentrated to approximate 15 mg/ml. Protein concentrations were determined by absorbance at 280 nm, assuming an $A_{280}$ of 0.506 for a 1.0 mg/ml solution. The initial screening conditions of native protein were Crystal Screen and Crystal Screen 2 from Hampton Research [12]. The crystallization experiments were conducted using the hanging-drop vapor-diffusion method at 20 ºC [13], with 2 μl drops containing 1 μl 10 mg/ml protein and 1 μl reservoir solution suspended over 0.5 ml reservoir solution. The crystallization condition was optimized at 4 ºC and 20 ºC by varying the type and concentration of precipitates (PEGs), pH, the kinds of additives and the protein concentration. The crystallization of SeMet derivate of CutC was performed by cross-seeding, micro-seeding and macro-seeding in the same conditions as employed for the native crystals.

### 2.4. Data Collection and Processing

All diffraction data were collected at the beamline 6A at the Photon Factory (Tsukuba, Japan) using an ADSC Quantum-4 CCD detector. All crystals were briefly soaked in the paraffin oil (Hampton Research) after being mounted in nylon cryoloops (Hampton Research) and then flash-cooled in a nitrogen-gas stream at 95 K. The native data set was collected at a wavelength of 0.9780 Å with a 150mm crystal-to-detector distance and 0.5º oscillation per frame. Each frame was exposed for 30 s and the total oscillation range covered was 110º. A single SeMet derivative crystal was used to collect MAD data at three different wavelengths. The optimal wavelengths for data collection were chosen after recording an X-ray fluorescence spectrum. The wavelengths selected were 0.97938 Å for the inflection point of the fluorescence spectrum (f' minimum), 0.97850 Å for the peak (f" maximum) and 0.9700Å for the remote high-energy point. The MAD Data were collected under the same conditions: the crystal-to-detector distance of 180 mm, the oscillation angle of 1º, the exposure time per frame of 20 s and the total oscillation range of 280º. MOSFLM (V6.2.2) [14], TRUNCATE and SCALA from CCP4 program suite V.4.2.2 [15] were used for processing, reduction and scaling of the diffraction data.

## 3. RESULTS

In the initial screening, small block-shaped native crystals were observed in Crystal Screen No. 46 (18%PEG8000, 0.1M sodium cacodylate pH6.5, 0.2M calcium acetate) after two days. As the results of optimization, the block-shape native crystals could grow up from these refined conditions. However, the growing crystals are always in accompanying with heavy precipitates and diffract very poorly (below 5 Å). Micro-seeding and macro-

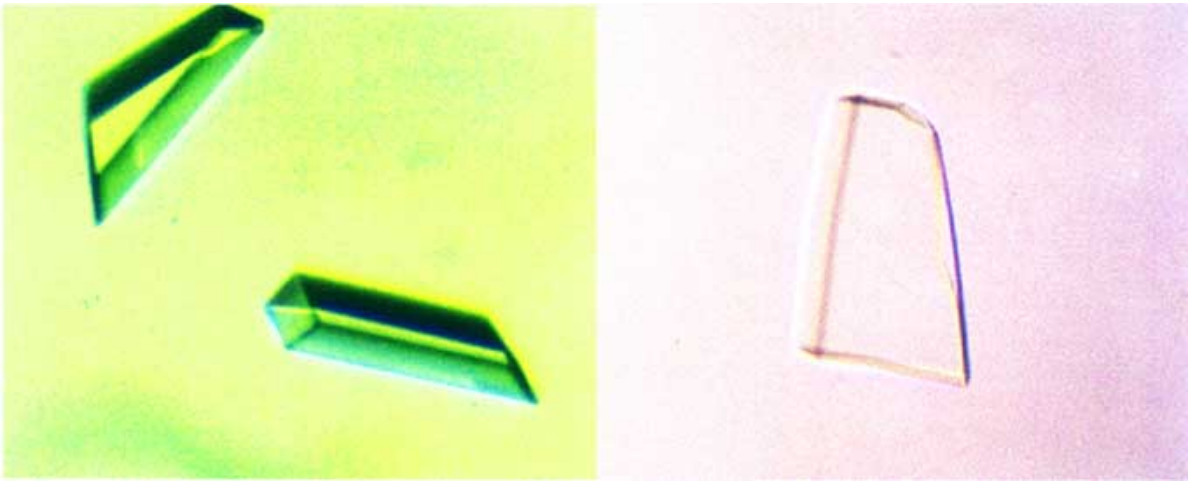

**Figure 1. a.** Crystals of native CutC protein. **b.** Crystal of SeMet CutC protein.

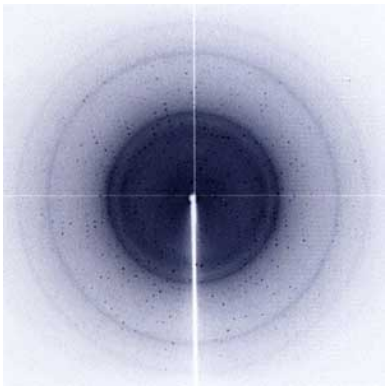

**Figure 2.** The image of the native CutC diffraction pattern. The crystal diffracted to a resolution of 1.7Å.

seeding were combined as a whole system to optimize the growth of the native crystals, and the qualified native crystals (0.15_0.07_0.05) (Figure **1a**) were obtained by the whole seeding method with the recipe of 6 mg/ml protein equivalent with the optimization reservoir solution: 20% PEG8000, 0.1M sodium cacodylate pH 6.1, 0.15M calcium chloride. The qualified crystals of the SeMet-derivate (0.1_0.1_0.06) (Figure **1b**) were obtained by cross-seeding (the seeds come from the native crystal) and the reservoir solution was 20% PEG8000, 0.1 M HEPES-Na pH 7.0 and 0.1 M calcium acetate.

The Data-collection statistics of native and SeMet-derivate crystals are shown in **Table 1**. The native crystal diffracted to the resolution of 1.7Å (Figure **2**). Crystals proved to be orthorhombic (C2221), and the native crystals cell parameters are: a=75.3267, b=97.6718, c=132.6910, $\alpha=\beta=\gamma=90^{\circ}$. Theses data are compatible with the presence of two molecules in the asymmetric unit and a solvent content of 43.89% (Vm= 2.19Å$^3$Da$^{-1}$; Vm: volume mass). The SeMet-derivate crystal has the diffraction resolution of 2.1 Å and belongs to the same space group and similar cell dimension of the native crystal.

## ACKNOWLEDGEMENTS

This work was supported by Grants from the Ministry of Science and Technology (G19990756, 2003BA711A13), the Chinese Academy of Sciences (KSCX-SW-17) and the NNSF (30370320) of China. The X-ray data collection was supported by the Photon Factory, KEK, Japan (00G209). We

**Table1. Statistics of X-ray data sets of native and SeMet crystals. (Values in parentheses refer to the highest resolution shell.)**

| Data set | Native | SeMet | | |
|---|---|---|---|---|
| | | edge | Peak | remote |
| Resolution(Å)[a] | 45.83-1.70 (1.79-1.70) | 59.26-2.10 (2.21-2.10) | 59.26-2.10 (2.21-2.10) | 59.26-2.10 (2.21-2.10) |
| Wavelength(Å) | 0.9780 | 0.97938 | 0.97850 | 0.9700 |
| No. of unique reflections | 52869 | 28435 | 28370 | 28481 |
| Completeness (%)[a] | 97.9 (91.7) | 99.6 (97.5) | 99.6 (97.6) | 99.8 (98.4) |
| Redundancy [a] | 4.4 (3.6) | 10.4 (7.0) | 10.5 (7.1) | 10.6 (7.4) |
| Average I/α(I) [a] | 8.2 (4.4) | 8.0 (3.4) | 8.3 (4.3) | 8.1 (4.0) |
| Rmerge(%)[a] | 5.7 (15.9) | 7.6 (20.9) | 7.1 (16.4) | 7.3 (18.1) |

[a] Values in parentheses refer to the outer resolution shell.

thank Professors N. Sakabe and F. Jiang for their help during data collections.

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Received on August 18, 2004, accepted on September 17, 2004