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**Understanding the role of SILE in the production of metal nanoparticles by *Morganella psychrotolerans* using MicroScale Thermophoresis**

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## **Abstract**

Metal nanoparticle synthesis has been observed in several species of bacteria but the underlying mechanisms of synthesis are not well understood. *Morganella psychrotolerans* is a Gram-negative psychrophilic bacterium that is able to tolerate relatively high concentrations of Cu and Ag ions, and it is through the associated resistance pathways that this species is able to convert metal ions to nanoparticles. The purpose of this study was to investigate the mechanism of nanoparticle synthesis, looking at the interaction of the metal binding protein SilE with metal ions using MicroScale Thermophoresis (MST). MST assays give a rapid and accurate determination of binding affinities, allowing for the testing of SilE with a range of environmentally significant metal ions. The binding affinities of Ag<sup>+</sup> and Cu<sup>2+</sup> were measured at 0.17 mM and 0.13 mM respectively, consistent with the observations of strong binding reported in the literature, whereas the binding to Al and Co ions was measured as a dissociation constant of 4.19 mM and 1.35 mM respectively.

## **List of abbreviations**

MST, MicroScale Thermophoresis; K<sub>d</sub>, Dissociation constant

## **Introduction**

Metal homeostasis is essential for the survival of all organisms [1]. While metal ions such as calcium, copper, chromium, nickel and zinc are required for a variety of vital cellular processes [2, 3] when in excess they become toxic to the cell, leading to cell death. To counter metal accumulation in the cell, bacteria have evolved intricate systems to maintain the correct internal concentration of metal ions [4, 5].

The large variety of environmental metals and their different physicochemical characteristics mean that numerous metal resistance mechanisms are required. Each mechanism is therefore specific to one or a small number of metals with similar characteristics. Such systems include the widely studied *Mer* pathway that reduces ionic  $\text{Hg}^{2+}$  to  $\text{Hg}^0$  in some *Trichoderma* strains [5], the Gram-negative based Cu tolerance system composed primarily of the copper sensor protein CueR and copper export pump CopA [6], and the *Cus* pathway in *E. coli* that is specialised in exporting Cu ions [5, 7].

One method of metal resistance is the conversion of a toxic ionic form of a metal into a relatively inert metal nanoparticle form [8]. This precipitated metal state removes the metal from the solution, preventing interaction with cellular components, and the process thereby provides the organism with resistance against those metal ions it is capable of transforming into nanoparticles [7]. *Delftia acidovorans* utilises this method, producing the extracellular protein delftibactin, which reduces Au ions into gold nanoparticles that are less reactive [9]. *Desulfovibrio alaskensis* G20 is able to reduce platinum group metals such as Pt and Pd, with the reduction occurring on the cell surface, resulting in nanoparticles attached to the outer cell membrane and thereby limiting uptake of toxic metal ions [10].

*Morganella psychrotolerans* is a Gram-negative psychrotolerant bacterium that is able to grow at a temperature range of 2-35°C [11]. It is known for its resistance to high levels of Ag and Cu [12, 13] and in previous studies, *M. psychrotolerans* has been observed to produce Cu and Ag nanoparticles [12, 14]. The similarity of these metals (both Ag and Cu have a comparable electron configuration, resulting in similar chemical behaviour) may mean that they share the same resistance/nanoparticle synthesis pathway. As Ag and Cu nanoparticles have the potential to be used in a wide range of industrial applications, such as anti-microbial agents, catalysts and in microelectronics [15, 16], knowledge of how *M. psychrotolerans* synthesises these nanoparticles will allow one to engineer this organism to produce tailored,

industrially useful nanoparticles. The first obstacle to overcome is nanoparticle yield; our previous research has shown that, although *M. psychrotolerans* synthesises Ag and Cu nanoparticles, the yield is reasonably low (data not shown). By understanding the role and characteristics of each protein involved in the resistance/nanoparticle synthesis pathway, it is easier to choose the best targets for engineering a strain with an increased nanoparticle yield, broader synthesis profile or increased selectivity. Metals such as Al, Cd, Mn, Co, Li and Zn are all of environmental and technical importance [17, 18] and have therefore been included in this study.

One of the proposed components of the Cu/Ag resistance mechanism in *M. psychrotolerans*, SilE [7], was chosen and expressed in recombinant form in *Escherichia coli*. SilE is a small periplasmic protein involved in the removal of excess  $\text{Ag}^+$  from the cell. It acts as a metal chaperone, binding to  $\text{Ag}^+$  and transporting it from a P-Type ATPase (SilP) to the SilCBA complex for subsequent export [19]. A homologue of SilE has also been observed to bind divalent ions such as  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$ , however to the best of our knowledge no binding affinities have been reported in the literature [19]. SilE is similar to PcoE and CusF in that they all bind to  $\text{Cu}^+$  and  $\text{Ag}^+$  ions and act as chaperones, transporting these ions within the periplasm for their subsequent removal from the cell through their respective pathways. SilE has also been found to be 48% homologous to the PcoE on the amino acid level, further suggesting a common function [20].

In order to investigate the possibility of other metals sharing the same resistance/nanoparticle formation pathway as Ag and Cu, the selectivity of SilE was tested by measuring its binding affinity for a range of metal ions using MicroScale Thermophoresis (MST).

## Materials and Methods

### *Plasmid construction*

The *silE* gene fragment from *M. psychrotolerans* was amplified by PCR with primers silE-F (5'CTAGTCCCATGGCTAAAAGCATTATGAAAAAAGT'3) and silE-R (5'GACTAGAAGCTTACTCTGTCTGCGGTGTTGTT'3) using purified *M. psychrotolerans* genomic DNA as the template. The SilE-F primer included an *NcoI* restriction site; to compensate for the frame shift of the start codon, two nucleotides (CT) were added immediately downstream of the *NcoI* site, resulting in the addition of an alanine residue to the N-terminus of the protein sequence. The primer silE-R removes the stop codon of *silE*, allowing incorporation of the TEV cleavage site and His<sub>6</sub>-tag (followed by a stop codon) found in the destination vector. The PCR product was inserted into the pET28a vector by digesting both with *NcoI* and *HindIII*, ligating with T4 ligase. The assembled plasmid was transformed into *E. coli* BL21 (DE3) cells. Transformed cells were plated onto Luria broth (LB) agar plates containing kanamycin (50 µg/ml) as the selection marker. The sequence of the construct was verified by Sanger sequencing.

### *Expression and purification of silE*

To induce protein production, 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG), was added to a log phase culture of *E. coli* (pET28a::*silE*) and incubated at 37°C for 4 h at 200 rpm in LB with kanamycin (50 µg/ml). At the end of the incubation, the culture was pelleted and sonicated in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4). Centrifugation at 3,894 x g for 10 min (Sigma 3-16KL centrifuge) was used to pellet cell debris. The supernatant containing SilE was collected and filtered through a 0.22 µm syringe filter (Millex-GP, Merck).

A 1 ml nickel column (HisTrap FF, GE Healthcare), mounted on an ÄKTAprime plus (GE Healthcare), was used to purify SilE-TEV-His (Affinity Purification any HiTrap template). The fractions were collected and analysed for purity, with the best being pooled. The pooled fractions were transferred into a Vivaspin® 20 (GE Healthcare), 10 kDa molecular weight cut-off polyethersulfone column and buffer exchange carried out. One column volume of PBS was added and centrifuged at 3,984 x g for 40 min; this was repeated for a total of three times. The protein was concentrated to a final volume of 0.5 ml and stored at 4°C.

#### *Removal of His<sub>6</sub>-tag*

As the His<sub>6</sub>-tag is able to bind metals, it had to be removed from SilE in order to test binding affinities of metal ions. TEV protease (Sigma-Aldrich) ( $\geq 2$  mg/ml in 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM TCEP, and 50% glycerol) was added to SilE in a 1:50 (w/w) ratio and incubated at 30°C for 2 h. The cleaved protein was then purified once more to remove free His<sub>6</sub>-tags and any SilE that retained a tag using HisPur™ Ni-NTA Resin (Thermo Fisher Scientific). The concentration of the purified protein was determined using a Bradford assay (Quick Start Bradford Protein Assay, Bio-Rad).

#### *Protein labelling*

SilE labelling was performed using the Monolith NT Protein Labelling Kit RED-NHS according to the manufacturer's (NanoTemper, MO-L001) instructions which labels primary amines present on lysine residues as they are more readily accessible by solvents. The protein concentration was adjusted to 20  $\mu$ M and 100  $\mu$ l used for the labelling procedure. The excess dye was then washed out and the resulting labelled protein was divided into 10  $\mu$ l aliquots and frozen at -80°C.

#### *Binding affinity assays using MST*

Binding affinities were measured using a Monolith NT.115Pico (NanoTemper) that features high sensitivity for detection at the pmol range. The metals tested were Al, Ag, Cd, Co, Cu, Li, Mn and Zn. For each metal, 100 nM of labelled SilE in MST buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.05 % Tween-20) was used. A dilution series of each metal was prepared, with the highest concentration being 10 mM. The assays were run according to the manufacturer's instructions with three replicates.

## Results and Discussion

### *MST assays*

MST has enabled valuable insights into the binding affinities of the protein SilE. Assuming that the heat-induced changes in diffusion detected by MST reflect direct binding of a single metal ion to the protein, SilE showed a good affinity to Ag<sup>+</sup> and Cu<sup>2+</sup>, with a dissociation constant, K<sub>d</sub>, of 0.17 mM and 0.13 mM respectively (**Figure 1A** and **1B**). The binding affinity was statistically significant with a K<sub>d</sub> confidence of 6.0x10<sup>5</sup>, 4.0x10<sup>5</sup> and 6.0x10<sup>5</sup> for Ag<sup>+</sup> and 4.2x10<sup>5</sup>, 3.2x10<sup>5</sup> and 4.0x10<sup>5</sup> for Cu<sup>2+</sup> (3 replicates for each metal). This is consistent with the literature; it has been well established that SilE binds to both metals, and it was hypothesised to be in the μM range [21]. The comparable dissociation constants between the two metals may be due to their similarity, being in the same chemical group, and thus behaving in a similar way while acting as ligands to the protein. Al<sup>3+</sup> and Co<sup>2+</sup> were also observed to bind to SilE, albeit more weakly (**Figure 1C** and **1D**), with dissociation constants of 4.19 mM and 1.35 mM respectively. The binding affinity was statistically significant with a K<sub>d</sub> confidence of 3.0x10<sup>3</sup>, 8.4x10<sup>4</sup> and 1.8x10<sup>3</sup> for Al<sup>3+</sup> and 6.2x10<sup>4</sup>, 3.0x10<sup>4</sup> and 6.1x10<sup>4</sup> for Co<sup>2+</sup> (3 replicates for each metal). As these are an order of magnitude weaker than the



affinities of Ag and Cu, the quantitative data suggest that SilE, and the metal resistance/nanoparticle production pathway it is part of, are specific to only a few metals.

A range of other metals were tested as ligands for SilE in order to see how selective this protein is.  $\text{Cd}^{2+}$ ,  $\text{Li}^+$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$  were analysed in their ionic state, dissolved in pure water, with all showing no binding affinity for SilE under the conditions used here (**Table 1**), again highlighting the specificity of the pathway.

Previous research has shown that *M. psychrotolerans* produces Ag and Cu nanoparticles [12-14], whilst there are no reports of the synthesis of other metal nanoparticles with this organism. Indeed, in our work with *M. psychrotolerans* we have been unable to detect any nanoparticle formation for Al, Cd, Co, Li, Mn and Zn. This current study bears out the fact that, while *M. psychrotolerans* may have mechanisms to confer resistance to these metals, they are most likely unrelated to the mechanism that includes SilE and results in nanoparticle formation. Thus implying that *M. psychrotolerans* is only able to synthesise specific metal nanoparticles using the *Cus* metal resistance system.

## **Conclusion**

Although the microbial synthesis of metal nanoparticles has been widely studied in recent years, the exact mechanisms that underlie their formation are not well understood. Here we attempt to understand more about the selectivity of SilE to a range of metals using MST. The results obtained from MST experiments showed that SilE derived from *M. psychrotolerans* binds monovalent Ag and Cu ions with good affinity. Other proteins involved in this pathway will need to be explored for their binding affinity to these metals, in isolation and together, to further understand the interactions between them in order to get a clearer understanding of the metal nanoparticle synthesis pathway.

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## **Author Contributions**

The manuscript was written through contributions of both authors. Both authors have given approval to the final version of the manuscript. N.P. and L.H. designed experiments. N.P. performed the experiments. N.P. and L.H. wrote the manuscript.

## **Conflicts of interest**

None.

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## **Legends to figure and table**

**Figure 1:** Dose response titration is shown for each metal that was found to bind with SilE. Detection of metal binding to SilE was determined by measuring fluorescence intensity at a range of metal concentrations to a fixed amount of substrate. The change in MST signal was fitted to the fluorescence intensities to yield a  $K_d$  value for each metal tested.

**Table 1:** Summary of the dissociation constants obtained in MST experiments with SilE and a range of metals.