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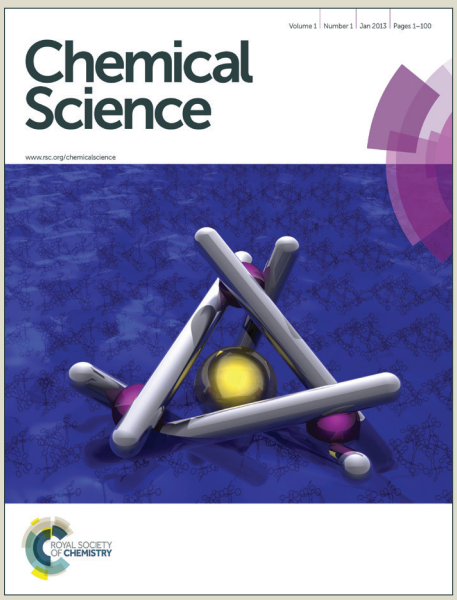

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ARTICLE TYPE

Biosynthesis of Silver Nanoparticles from Silver(I) Reduction by the Periplasmic Nitrate Reductase c-type Cytochrome Subunit NapC in a Silver Resistant *E .coli*

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The synthesis of metal nanoparticles by using bacteria is of growing interest in nanobiotechnology as well as in the study of microbial metal metabolism. Some silver resistant bacteria can produce considerable amount of silver particles when exposed to silver salts at high concentration but the mechanism of biosynthesis is unknown. In this work, an *E. coli* strain that carries chromosomally encoded silver resistance determinants has been shown to produce silver nanoparticles in the periplasmic space when it was exposed to Ag(I) salts, providing a prototypical model for studying the biosynthesis of silver nanoparticles. The synthesized silver nanoparticles are in the form of zero-valent metallic silver lattice and the production of which was observed to be favorable under anaerobic conditions, suggestive of biological reduction of Ag^+ ions. As the microbial c-type cytochromes are known to mediate respiratory reduction of metal ions, their role in the biosynthesis of silver nanoparticles was examined. A deletion mutant of the cytoplasmic membrane-anchored tetra-heme c-type cytochrome subunit of periplasmic nitrate reductase (NapC) showed markedly reduced production of silver nanoparticles. On the other hand, re-introduction of the NapC could recover the biosynthesis of the silver nanoparticles. This study has identified a molecular mechanism of biosynthesis of silver nanoparticles involving c-type cytochromes, having implication in bioenvironmental process of mineralization and synthetic biology of metal nano-materials.

Introduction

Microorganisms can change the oxidation state of metal/metal ions through metabolic processes.[1-3] Certain metal-reducing microbes could conserve energy by making use of oxidized metal ions as terminal electron acceptors during anaerobic respiration, while carrying out dissimilatory reduction converting the insoluble oxidized metal ions [e.g. Fe(III) and Mn(III,IV)] into soluble reduced forms [e.g. Fe(II) and Mn(II)].[4-6] A notable metal-reducing bacteria is the *Shewanella oneidensis* MR-1 which can reduce many transition metal ions including Fe(III), Mn(III,IV), Cr(VI), U(VI), Se(IV) and V(V) by an array of multi-heme c-type cytochromes.[7, 8] These biological metal reduction processes are not only important in natural metal cycles but are also of interest in bioleaching processes of ores and bioremediation of polluted environment.[9] The microbial reduction of metal ions could also result in accumulation of metal in the microbial biomass and culture. Recently there is an upsurge of interest in the microbial reduction of metal ions to metal nanoparticles that are potentially useful in technological applications such as catalysis, photonics and in biological recovery of metals.[10-14]

The microbial reduction of precious metal ions, such as that of palladium, platinum, silver and gold into metallic nanoparticles have been known in literature.[15] As these metal ions are toxic to the micro-organisms, biological resistance mechanisms[3] are required for efficient biosynthesis of the metal particles. In particular, some silver resistant bacterial strains isolated from silver mines have been shown to produce significant amount of silver nanoparticles (nano-Ag) when exposed to silver salts. [16, 17]Nonetheless, little is known about the molecular mechanism(s) involved in microbial production of silver nanoparticles. In this work, we have characterized the mechanism of intracellular synthesis of nano-Ag in a silver resistant *E. coli* strain [17] that displays robust resistance to silver owing to the constitutive expression of cusCFBA copper/silver efflux system.[18, 19] This silver resistant bacterial strain accumulates nano-Ag in the periplasm upon prolonged exposure to silver nitrate at high concentrations. The biosynthesis of nano-Ag is favored under anaerobic stationary growth condition and is inhibited by cyanide and nitrate. Inspired by the robust capacity of metal reducing bacteria such as *S. oneidensis* MR-1 which utilize multi-heme c-cytochromes for the reduction of metal ions including Ag^+ ions,[7, 8, 20] we have examined the role of the c-type cytochromes of the silver resistant *E. coli* strain in the biosynthesis of nano-Ag. Deletion mutant analysis of the silver resistant bacteria revealed that the membrane-anchored tetra-heme c-type cytochrome subunit of the periplasmic nitrate reductase (NapC)[21, 22] resulted in marked decrease in the nano-Ag accumulation. This study identified a molecular mechanism of biosynthesis of silver nanoparticles that has implication in bioenvironmental process and in the production of metal nano-materials by synthetic biology approach.

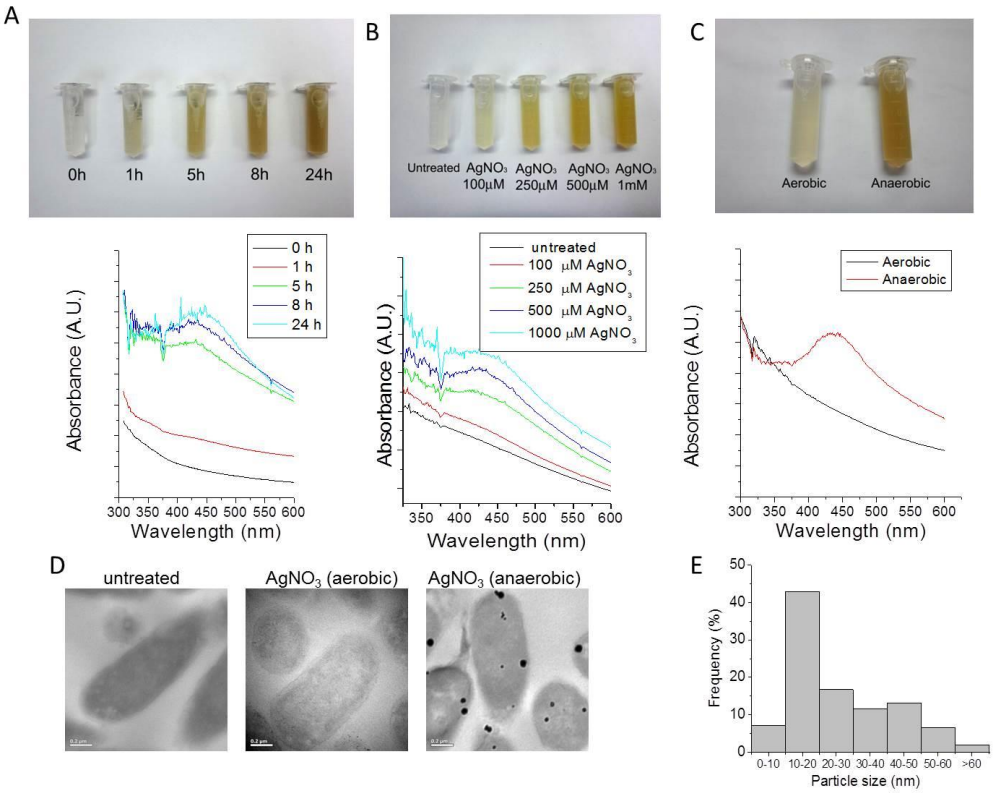

Fig. 1 Biosynthesis of nano-Ag by 116AR *E. coli* Silver Resistant Strain. A. Cells were cultured in M9 medium with 300 μM $AgNO_3$ at time period. The appearance of culture and the absorption spectrum of the culture were shown. B. Cells were treated with various concentrations of $AgNO_3$ for 18 h under anaerobic condition. C. Cells were treated with 300 μM $AgNO_3$ for 18 h under aerobic or anaerobic condition. D. TEM of ultrafine sections of cells. E. Nano-Ag particle size distribution.

Results

An *E. coli* strain (116AR) that can maintain normal growth in the presence of milli-molar concentrations of Ag^+ was isolated by Li *et al* using procedures of stepwise selection against increasing concentrations of silver nitrate.[18] Our previous study indicated that the silver resistance is mainly conferred by the constitutively expressed chemiosmotic copper efflux system CusCFBA which mediates active efflux of silver ions.[19] When this silver resistant strain (116AR) was cultured in the presence $AgNO_3$ under anaerobic condition overnight, the color of the culture turned intensely amber, showing an absorbance peak at ~ 440 nm superimposed on broad shoulder of turbidity absorbance of the cell suspension. The amber coloration could be an indication of formation of nano-Ag which has typical surface plasmon resonance (SPR) absorption at around 400-450 nm.[23] The absorbance attributed to nano-Ag was evident at 8 h, became maximal after overnight incubation in the presence of 500 μM $AgNO_3$ and was dependent on the applied $AgNO_3$ concentrations from 100 μM to 1 mM **(Fig. 1A & 1B)**. Under aerobic condition with sufficient aeration, the $AgNO_3$-treated bacterial culture did not significantly turn color as compared with that grown under anaerobic condition **(Fig. 1C)**.

Transmission electron microscopy (TEM) revealed that the silver-treated 116AR bacteria had nano-sized electron-dense spherical particles deposited predominantly in the periplasmic space of bacterial cells **(Fig. 1D)**. The particle size were in the range from 5 nm to 70 nm with an average size of 26.9 nm **(Fig. 1E)**. The identification of nano-Ag was revealed by energy-dispersive X-ray spectroscopy (EDX) analysis **(Fig. 2A)**. The EDX result also indicated the presence of P, Cl and S signals in the accumulated particles, all of which were also detected in other area besides the particles. Based on selected area electron diffraction (SAED) pattern, the nano-Ag were in highly crystalline pattern with the interplanar d-spacings of four ring patterns are 0.246, 0.210, 0.148, 0.125 nm, all of which are in accord to that of zero-valent silver (PCPDF number: 04-0783) **(Fig. 2B)**. TEM analysis of the bacteria also showed that the accumulation of nano-Ag inside the bacterial cells was markedly suppressed in the aerobic culture **(Fig. 1D)**. The nano-Ag deposited in the bacterial periplasm was extracted by osmotic shock procedures. The nano-Ag showed SPR absorbance at 416 nm **(Fig. 2D)** that was comparable to the SPR absorbance of nano-Ag prepared from $NaBH_4$ reduction of silver nitrate **(Fig. 2E)**. The silver content of the particles was confirmed by TEM followed by EDX analysis **(Fig. 2C)**.[23]

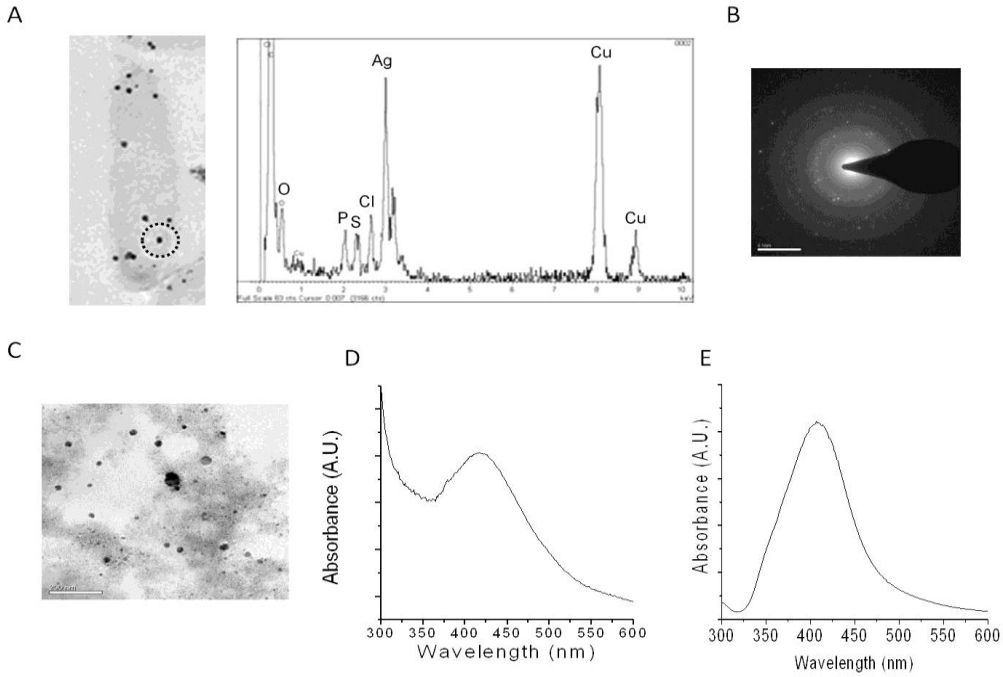

Fig. 2 Characterization of the Periplasmic nano-Ag Synthesized in the 116AR *E. coli.* A. EDX analysis of the nano-Ag deposited in the cells (circled). B. The selected area electron diffraction (SAED) pattern of the deposited nano-Ag. C. TEM images of nano-Ag isolated from the periplasmic fraction. D. SPR absorption of nano-Ag isolated from the periplasmic fraction. E. SPR absorption of chemically synthesized nano-Ag by borohydride reduction.

Attempts were made to elucidate the molecular mechanism involved in the biosynthesis of nano-Ag in the silver resistant bacteria. With reference to the dissimilatory metal reducing bacteria (DMRB) such as *Shewanella oneidensis* MR-1 which employs c-type cytochromes for reduction of exogenous metals,[7, 22] the role of several *E. coli* c-type cytochromes in the course of biosynthesis of nano-Ag under anaerobic condition was examined. Genes encoding oxidoreductases containing the c-type cytochromes subunits (NapB, NapC, NrfA, NrfB, NarI, NarV, TorC and TorY) and located in the inner membrane or periplasmic space (www.ecocyc.org) were selected for the analysis. The respective c-type cytochromes deletion mutants of the 116AR strain were constructed by P1 transduction. The bacterial cultures of these strains were grown overnight to stationary phase under anaerobic condition and replenished with the same volume of fresh M9 medium. Cells were then exposed to 300 μM $AgNO_3$ for 18 h under anaerobic condition and the nano-Ag production was followed. Except for NapC deletion mutant, all mutants turned amber in color indicative of the nano-Ag accumulation **(Fig. 3A)**. The Δ*napC* mutant displayed much pale coloration in the saturated culture. As depicted in **Fig. 3B**, while there was significant nano-Ag production in the 116AR cells after silver treatment, the Δ*napC* mutant did not. To further confirm the role of NapC in the biosynthesis of nano-Ag, the experiments were performed with re-introduction of NapC in the Δ*napC* mutant by transformation of a lac promoter –driven NapC expression plasmid. In the 116AR Δ*napC* cells with induced NapC expression, partial but marked recovery of nano-Ag synthesis as revealed by the SPR absorption and TEM analysis was observed **(Fig. 3B & 3C)**.

There are additional biochemical evidences supporting the c-type cytochromes such as NapC as the mediator of nano-Ag biosynthesis. Cyanide ion has a high affinity to ferric heme, inhibiting the redox cycling of cytochromes and blocking the cytochrome-mediated electron transfer in the electron transport chain. [24] When 116AR strain was pre-treated with 25-100 μM of sodium cyanide followed by exposure to excess $AgNO_3$, the biosynthesis of nano-Ag was significantly suppressed as indicated by the decrease in the absorbance of the 440 nm absorption peak **(Fig. 4A)**. As shown by the TEM analysis, the elemental silver deposition in the cyanide treated cells was found to be markedly decreased compared to the untreated control **(Fig. 4C)**.

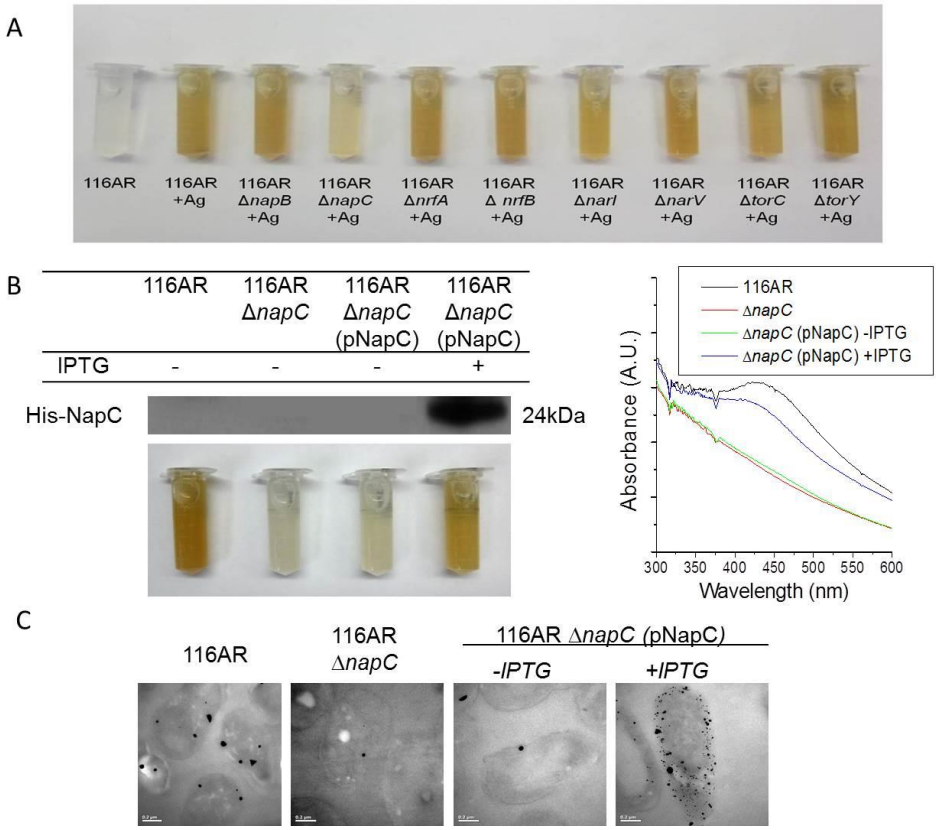

Fig. 3 Identification of Periplasmic Nitrate Reductase c-type Cytochrome Subunit NapC as the Metal Reductase for Biosynthesis of nano-Ag in 116AR *E .coli*. A. Appearance of saturated overnight culture of 116AR and the cytochrome deletion mutants grown in the presence of 300 μM $AgNO_3$ under anaerobic condition. B. Expression of NapC in 116AR Δ*napC* strain. 116AR Δ*napC* was transformed with lac promoter-driven His-tagged NapC plasmid (pNapC). The NapC expression was induced by IPTG and confirmed by immunoblot using His-tagged antibody. Stationary phase culture of the 116AR, 116AR Δ*napC* and the 116AR Δ*napC* with re-introduced NapC expression were treated with 300 μM $AgNO_3$ for 18 h. The appearance of culture and the absorption spectrum of the culture were recorded. C. TEM images of the corresponding samples in B.

Nap is the nitrate reductase system located in periplasm, containing Nap G, H, C, B & A subunits. [21, 22] The expression of Nap proteins is induced by anaerobiosis and suppressed by high concentrations of nitrate.[25] When the 116AR strain was treated with nitrate and incubated under anaerobic condition, the nano-Ag accumulation was decreased as revealed by loss of the nano-Ag SPR absorbance upon increasing the nitrate concentration from 10 mM to 50 mM **(Fig. 4B)**. TEM analysis also revealed marked reduction of intracellular silver deposit in the nitrate treated cells **(Fig. 4C)**.

Discussion

Nano-Ag could be produced by culturing the silver resistant 116AR *E. coli* strain in medium supplemented with high concentrations of $AgNO_3$. The spectral properties of the biologically synthesized nano-Ag shed information on the physical state of the nanoparticles. The 416 nm absorption peak of the biogenic nano-Ag is similar to the SPR absorption of chemically synthesized nano-Ag **(Fig. 2D & 2E)**. The findings of EDX and SAED experiments ascertained that the silver deposition was zero-valent metallic silver **(Fig. 2A & 2B)**.

When a diluted or saturated culture of the parental silver sensitive strain 116S was exposed to $AgNO_3$ solution, the bacteria was rapidly killed and no nano-Ag production could be observed. This finding showed that the synthesis of nano-Ag in the silver resistant strain requires live bacteria with a silver resistant background. As demonstrated by TEM and SAED analysis, the zero-valent nano-Ag accumulated in periplasmic space of 116AR, implying that the reduction reaction may be mediated by periplasmic oxidoreductases that are capable of reducing metal ions **(Fig. 1D)**. The bacterial production of nano-Ag is favored under anaerobic condition, suggesting that a reductive process takes place presumably by anaerobically induced enzymes **(Fig. 1C & 1D)**.

In nature, dissimilatory metal-reducing bacteria, such as those of *Shewanella* and *Geobacter* species, have evolved mechanisms for utilizing inorganic minerals as terminal electron acceptors during anaerobic respiration. [4-6] *Shewanella oneidensis* MR-1, one of the well-studied dissimilatory metal reducing bacteria, has a battery of c-type cytochrome-containing oxidoreductases that shuttle

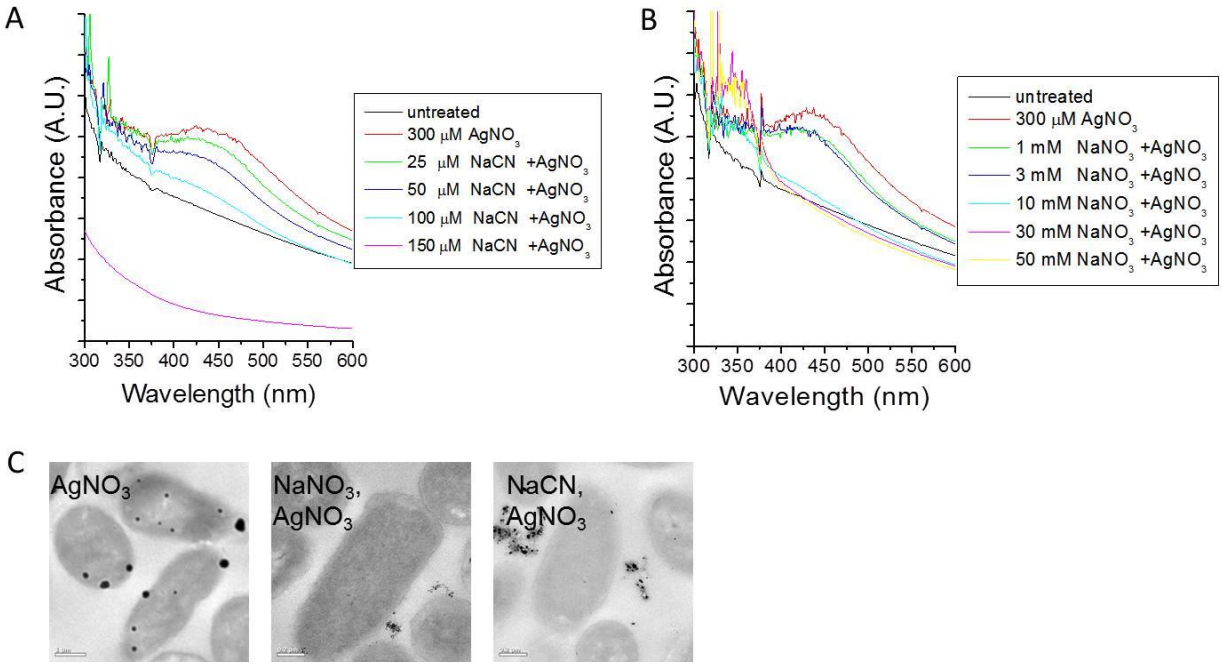

Fig. 4 The Biosynthesis of nano-Ag was Blocked by Cyanide and Nitrate in 116AR *E. coli*. Cells were treated with indicated concentrations (A) NaCN and (B) $NaNO_3$ followed by 300 μM $AgNO_3$ for 18 h. The absorption spectrum of the culture are shown. C. TEM images of cells treated with 30 mM $NaNO_3$ or 100 μM NaCN followed by 300 μM $AgNO_3$ for 18 h.

electrons from the intracellular quinol pool towards the outside of the cell during anaerobic respiration.[7] In particular, the tetra-heme *c*-type cytochrome protein CymA serves as a central hub for directing the electron flow from cytoplasmic membrane-bound menaquinol to several oxidoreductases located in the periplasm. [7, 8] In *E. coli*, there are several c-type cytochromes[22, 26] located in the inner membrane and in periplasm performing electron transfer in dissimilatory reduction of substrates including nitrate and nitrite under anaerobic conditions.[25, 27, 28] To assess the possible role of these c-type cytochromes containing oxidoreductases in the biosynthesis of periplasmic nano-Ag in the 116AR strain, deletion mutants of the respective cytochromes were prepared and their capability in synthesizing nano-Ag was examined. The findings indicated that the NapC deletion mutant had significantly weaker activity in nano-Ag production **(Fig. 3A)**, and re-introduction of NapC in the Δ*napC* mutant partially recovered nano-Ag synthesis **(Fig. 3B & 3C)**. These data suggest that NapC plays a key role in the nano-Ag synthesis in the silver resistant 116AR *E. coli* strain. In literature, there is some evidence for the involvement of outer membrane c-type cytochrome in the reduction of Ag^+ ions in *Geobacter sulfurreducens* but the enzymes involved have not been exactly identified.[29]

NapC is the inner membrane-anchored tetra-heme c-type cytochrome subunit of the periplasmic nitrate reductase, directing electron transfer from cytoplasmic membrane-bound menaquinol to periplasmic catalytic subunits NapAB. [21, 22] NapC shares high degree of similarity in amino acid sequence and size with CymA and has been shown to exhibit orthologous activity to the CymA of *Shewanella oneidensis* MR-1 in the reduction of ferric complexes.[22] Thus the biosynthesis of nano-Ag by NapC is compatible to its role in the direct reduction of metal species. It should be noted that residual nano-Ag production was still observed in the Δ*napC* mutant **(Fig. 3B & 3C)**, suggesting NapC is not the only enzyme that reduces silver ions into nanoparticles. We are aware that *napC* gene sequence appears to harbor a weaker promoter of the downstream cytochrome c maturation genes *ccm* that are normally activated by Fnr proteins acting upstream to the *nap-ccm* operon.[30] Thus polar effect of NapC disruption on cytochrome assembly could not be excluded. Nonetheless, our data showed that re-introduction of NapC in the Δ*napC* mutant partially recovered nano-Ag synthesis, revealing that NapC does play a role in the nano-Ag biosynthesis **(Fig. 3B & 3C)**. Furthermore, introduction of an expression plasmid encoding the full set of *ccm* genes (CcmA-H)[31] to the Δ*napC* mutant did not recover the loss of nano-Ag production (Fig. S1, ESI†).

There is also circumstantial evidence supporting the involvement of c-type cytochromes such as NapC in the nano-Ag production. The inhibition of nano-Ag synthesis by cyanide **(Fig. 4A & 4C)** which avidly coordinates to heme iron and blocks the electron transfer is indicative of the involvement of hemoprotein like cytochromes. [24] The reason for inhibition of nano-Ag

synthesis by nitrate **(Fig. 4B & 4C)** at high concentrations remains to be elucidated but may be related to the down-regulation of the expression of periplasmic nitrate reductase *nap* operon expression which is normally activated by anaerobiosis and nitrate at low concentrations.[25] Alternatively high concentrations of nitrate substrate may compete with the diffusible metal species, favoring electron flow from NapC to the NapAB which are the catalytic nitrate reductase subunits.

A major detoxicification mechanism of silver resistant bacteria is through metal efflux and periplasmic metal binding.[32-35] The 116AR silver resistant *E. coli* exhibits high constitutive expression of CusCFBA chemiosmotic copper/silver efflux system and maintains growth in the presence of high concentrations of silver. With active efflux and periplasmic binding of Ag^+ ions, the cytoplasm is protected from the toxicity induced by silver that would have been accumulated to lethal concentrations.[19] Our preliminary data revealed that the *napC* deletion mutant showed a transient suppression in cell colonial growth in the presence of silver salt (Fig. S2, ESI†). If the bio-reductive synthesis of nano-Ag could play a role in the silver resistance, it is reasoned that under anaerobic and stationary growth conditions, the c-type cytochromes including NapC may confer some degree of tolerance to silver by a fortuitous reduction of Ag^+ with deposition of less active nano-sized Ag^0 species. In literature, it has been demonstrated that *E. coli* expressing an engineered silver-binding periplasmic protein with the formation of nano-Ag exhibited tolerance to silver salt in culture.[36] Nonetheless, when compared to Cus system whose disruption results in almost complete loss of bacterial survival,[19] the silver tolerance mediated by the reduction of Ag^+ seems to be less significant.

Conclusion

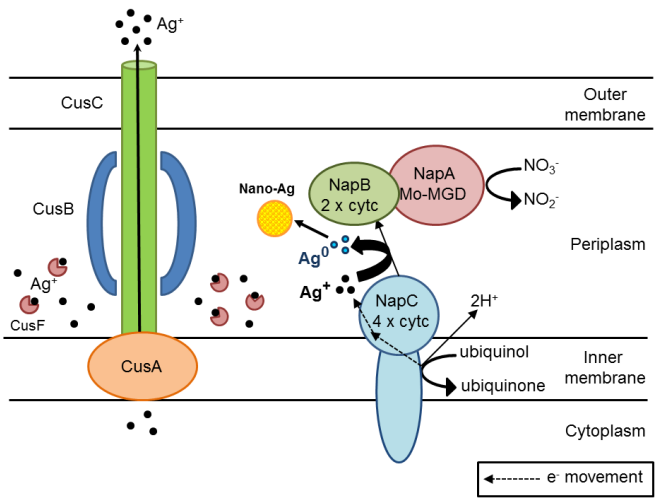

Fig. 5 A Proposed Model of Biosynthesis of nano-Ag by Periplasmic c-type Cytochrome NapC in the Silver Resistant *E. coli* Strain 116AR.

The proposed mechanism for the biosynthesis of silver nanoparticles in the silver resistant *E. coli* strain 116AR is depicted in **Fig. 5**. Upon exposure to Ag^+ ions, the CusCBA actively excludes Ag^+ ions from the cells and the periplasmic space appears to be a site at which Ag^+ ions concentrate. The metabolically viable silver resistant cells are capable of oxidizing respiratory substrate for electron transfer from quinol pool to the electron acceptor via the cytoplasmic membrane oxidoreductases. Under anaerobic condition, the c-type cytochromes such as NapC located in the periplasm can reduce the Ag^+ ions to nano-sized particles. The biological significance of the reductive deposition of nano-Ag in periplasm remains to be elucidated but may be related to partial silver tolerance in anaerobic growth conditions. The findings in the present study may help to understand the molecular step(s) involved in bio-geochemical silver accumulation in the environment where the silver resistant microbes inhabit (such as the *Pseudomonas stutzeri* in silver mine).[16, 17] The identification of specific c-type cytochromes and other molecular conduits for metal reduction will be useful for the preparation of metal-based nano-materials by synthetic biology approach.[37]

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Notes and references

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†Electronic Supplementary Information (ESI) available: Experimental procedures for bacteria, growth condition and protein expression, biosynthesis of silver nanoparticles, transmission electron microscopy, and energy dispersive X-ray analysis.

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