Synthesis, characterization and kinetic study of silver and gold nanoparticles produced by the archaeon Haloferax volcanii

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Keywords
antimicrobial activity, catalysis, gold nanoparticle, Haloferax volcanii, silver nanoparticle.

Abstract

Aims: To evaluate the ability of the haloarchaeon Haloferax volcanii to produce Ag and Au nanoparticles (NPs) and to characterize the obtained material in order to find relevant properties for future potential applications.

Methods and Results: Nanoparticles were produced by incubating H. volcanii cells with the corresponding metal salt. In the presence of precursor salts, cultures evidenced a colour change associated to the formation of metallic nanostructures with plasmonic bands located in the visible range of the spectrum. X-ray fluorescence analysis confirmed the presence of Ag and Au in the NPs which were spherical, with average sizes of 25 nmol l⁻¹ (Ag) and 10 nmol l⁻¹ (Au), as determined by electronic microscopy. Fourier transformed infrared spectroscopy indicated that both types of NPs showed a stable protein capping. Ag NPs evidenced antibacterial activity and Au NPs improved the specificity of polymerase chain reaction reactions. Au and Ag NPs were able to reduce 4-nitrophenol when incubated with NaBH₄.

Conclusions: Haloferax volcanii is able to synthesize metallic NPs with interesting properties for technological applications.

Significance and Impact of the Study: Our data demonstrate the ability of H. volcanii to synthesize metal NPs and constitutes a solid starting point to deepen the study and explore novel applications.

Introduction

Metals play a central role in the physiology of microorganisms and some of them, such as cobalt, chromium, calcium, iron, copper, potassium, magnesium, sodium, manganese, nickel and zinc, are required nutrients essential for life. The roles of essential metal ions are very diverse; they function as catalysts in biochemical reactions, stabilize protein structures and participate in maintaining osmotic balance (Nies 1999). In contrast, silver, gold, arsenic and mercury (among others) are not needed for microbial growth and are considered nonessential. These metallic ions are toxic for most microorganisms, and exert their toxicity by substituting essential metals from their natural binding sites or interacting with other specific ligands. At high concentrations, essential metals are also toxic, killing cells by disrupting cell membranes, altering enzymatic specificity or denaturing DNA (Hobman and Crossman 2015). When exposed to metals, microbes overcome the toxicity using various resistance mechanisms which are a result of enzymatic detoxification and/or extrusion of the metal through ion efflux pumps present in the cell. These defense mechanisms can be efficiently used by bacteria, fungi and archaea for the synthesis of metal nanoparticles (NPs), transforming toxic metals to their nontoxic nano-sized version or the soluble form to the insoluble nano-sized form (Das et al. 2014; Gahlawat and Choudhury 2019).
Metal NPs have unique traits due to their large surface to volume ratio which increases their interaction with other molecules, such as proteins, fatty acids, polysaccharides and nucleic acids (Zewde et al. 2016; Gahlawat and Choudhury 2019). NPs have potential applications in pharmacology, physics, cosmetics, chemistry, food and water safety, bioremediation, electronics and medical imaging (Kahn et al. 2012; Liu et al. 2014; Pereira et al. 2015; Ali et al. 2019).

Nanoparticles can be synthesized either by top-down or bottom-up strategies (Ahmed et al. 2016). In top-down strategies, the material is broken by physical methods into nano-sized particles using different techniques such as thermal/laser ablation, chemical etching or grinding. In the bottom-up approach, NPs are synthesized using chemical or biological methods. Chemical strategies include electrochemical precipitation, vapour deposition, seeded growth method, polyol synthesis method and pyrolysis. Biological methods imply the use of whole organisms or organism-derived compounds for synthesizing NPs. Both physical and chemical NP syntheses have a major downside, which is the toxic precursor chemicals used and the generation of hazardous by-products (Gour and Jain 2019). Biological synthesis of NPs using microorganisms and plants is a clean, nontoxic and an eco-friendly method and therefore is often referred as ‘green synthesis’. Additionally, the NPs synthesized by microorganisms are usually capped by stabilizing cellular metabolites produced during NPs generation. The synthesis may either be an intra- or extra-cellular process depending on the nature and the metabolic activity of the organism used. Numerous bacteria, fungi, algae and plants can serve as living nanofactories for the production of low cost and environmentally friendly metal NPs (Gour and Jain 2019; Gahlawat and Choudhury 2019), however, the biosynthesis of NPs using archaea has been scarcely reported (Beeler and Singh 2016).

Haloarchaea inhabit hypersaline environments including estuaries, solar salterns, salt lakes, brines and saline soils. Some of these ecologies may serve as sinks for metals that come from natural sources (i.e. river-borne) or from human activities (such as industry, urbanization and mining) (Srivastava and Kowshik 2013). Haloferax BBK2 and Halococcus BK6 display high Zn and Cd tolerance (minimal inhibitory concentration to inhibit 50% of growth (MIC50) of 0.5 mmol l⁻¹). Interestingly when grown in the presence of Cd salts, Haloferax sp. BBK2 accumulated Cd NP sintracellularly (Srivastava et al. 2014). In addition, Halococcus salifodinae BK18 was reported to synthesize selenium NPs (Das et al. 2014), and another strain, H. salifodinae BK3, was able to produce Ag NPs with the involvement of NADH-dependent nitrate reductase (Srivastava et al. 2013).

Halophilic organisms constitute a promising and relatively under-explored target for biotechnological applications due to the particular conditions their physiology requires (Singh and Singh 2017; Jia et al. 2017; Haque et al. 2019; Haque et al. 2020). The halophilic archaeon Haloferax volcanii has become an important archaeal model system, as it is an organism that can be easily cultivated in the laboratory and for which many biochemical, molecular and genetic tools have been developed (Pohlschröder and Schulze 2019). The aim of this work was to assess the ability of H. volcanii to synthesize metal NPs that could be applied, without exhaustive post-treatment, in technological processes. In the route to the implementation of biological synthesis as a reliable, useful and simple method for the design of nanostructures, their potentiality in diverse applications should be proved. In this work, besides demonstrating the ability of these haloarchaeon to synthesize metal NPs, we also characterized the obtained material and analysed the performance of these structures in three different scenarios: as antimicrobial agents, as catalysts in the reduction of 4-nitrophenol and as enhancers of the specificity of the polymerase chain reaction (PCR).

Materials and methods

Strains and growth conditions

The strain used in this work was H. volcanii H26 containing plasmid pTA963 (Allers et al. 2010). Cultures were grown in Hv-Min liquid medium (Dyall-Smith 2009) at 42°C and 150 rev min⁻¹ until stationary phase of growth was reached (3 days). Then, cells were harvested by centrifuging at 4800 g at 4°C for 10 min and the cell pellets were washed and suspended in buffer A (2 mol l⁻¹, 100 mmol l⁻¹ Tris-HCl, pH 7.5) for biogenic synthesis of NPs.

Escherichia coli K12 strain RP437 (Parkinson and Houts 1982) and Pseudomonas putida KT2440 were grown in Luria–Bertani (LB) medium at 37 and 30°C, respectively, at a stirring rate of 150 rev min⁻¹.

NPs biosynthesis

For the synthesis of Ag NPs, silver nitrate was added from a 0-1 mol l⁻¹ stock solution to the cells suspended in buffer A reaching the final concentrations indicated in each experiment, and then the mixture was incubated at 42°C from 1 h to overnight (ON) in darkness. The turbidity of the samples was due to the initial formation of AgCl. Further reduction of Ag(I) into Ag NPs produced a change in the colour of the culture from cloudy pink to brown. For cell disruption, cultures were sonicated...
(3 × 30 s) or, alternatively, cells were disrupted by the addition of water (osmotic rupture). Sonicated samples were centrifuged at 6700 g for 20 min and NPs-containing pellets were washed three times with buffer A and three times with bi-distilled water. Washing was followed by centrifugation at 6700 g for 10 min. Osmotically disrupted cells were washed with bi-distilled water (3x). NPs powder was obtained by vacuum drying the pellet during 2 h (SAVANT SpeedVac, Thermo Scientific, MA) or by drying at 42°C at 1 atm for 16 h.

Similar conditions were applied for the biosynthesis of Au NPs, using 0.0576 mol l⁻¹ chloroauric acid (HAuCl₄) as the initial metal salt. In this case, the culture medium changed from a light orange (due to the simultaneous presence of the reddish cells and the yellow Au precursor) to a purple-like colour as a consequence of the formation of Au NPs.

Characterization of NPs

The localized surface plasmon resonance of NPs was characterized by using UV–Vis spectrophotometry (GeneQuant1300; Biochrom, Cambridge, UK) in the range 300–700 nm. The presence of elemental Ag and Au was confirmed through X-ray fluorescence (XRF) analysis in a PW4025/24 Minipal2 X ray spectrometer (Panalytical) with a Cr anode (120 s, 10 µA and 20 KV). The morphology, shape, and size of NPs were observed by transmission electron microscopy (TEM) in a JEOLJEM-2100 microscope. For observation, 5 µl of the aqueous NPs dispersion was evaporated on a carbon coated copper grid (400 mesh). Field emission scanning microscopy (FESEM) images were obtained with a Zeiss Supra 40 SEM microscope. Samples were prepared by evaporating a drop of the dispersions on metal substrates. The functional groups responsible for the stabilization of Ag NPs were analysed by Fourier transformed infrared spectroscopy (FTIR). The IR spectra were obtained in ATR mode, in a Spectrum 100 spectrophotometer, from Perkin Elmer, with 4 cm⁻¹ resolution and 16 scans.

Proteomic analysis of protein corona by liquid chromatography coupled to mass spectrometry (LC-MS)

To identify the proteins associated to Ag NPs, polycrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) was performed. Ag NPs were incubated with 1% (p/v) SDS in a boiling water bath for 10 min. Untreated NPs were kept as control. Electrophoresis was performed in a 12% (p/v) SDS-polyacrylamide gel (Laemmli 1970). Samples were allowed to run (20 mA per gel) 1 cm and then the gel was stained with Coomassie Brilliant Blue dye and the stained band was excised from the gel and sent to a specialized centre for protein identification by tryptic digestion followed by LC/MS-MS (Cequibiem, Conicet-UBA, Argentina). The data obtained was analysed by searching against the complete proteome database of H. volcanii DS2 containing 4035 entries exported from the Halolrex database (Pfeiffer et al. 2008).

Antimicrobial activity

The antimicrobial activity of biosynthesized Ag NPs on E. coli and P. putida was examined using a plate diffusion method. Briefly, a drop of 10 µl of Ag NPs solution (1 mg ml⁻¹) was placed in soft (0-7% p/v) LB agar plates previously inoculated with 100 µl of 1 × 10⁹ bacterial suspension (grown ON in LB medium). Ampicillin (1 mg ml⁻¹ for E. coli and 100 mg ml⁻¹ for P. putida) and sterile bi-distilled water were used as positive and negative controls, respectively. The plates were subsequently incubated at 37°C (E. coli) and 30°C (P. putida) for 16 h, and the antimicrobial activity was analysed by measuring the diameter of the inhibition zone (mm) around the well. Results were analysed by GLM (general linear model) with normal distribution to determine statistical significance between treatment and controls (n = 9). A post hoc analysis was conducted with the ‘emmeans’ package (Lenth 2019). All analyses were performed using R software ver. 3.6.1 (R Core team 2019). Statistically significant differences were determined at P < 0.05.

Catalytic reduction of 4-nitrophenol

Catalytic activity of biosynthesized NPs was evaluated through the reduction of 4-nitrophenol in excess of NaBH₄. The experimental procedure and the starting operating conditions were initially set from conclusions arisen from a previous study in which the catalytic activity of Au NPs coated and uncoated with polyvinylpyrrolidone (PVP) was compared (ÁlvarezCerimedo et al. 2019). Hence, for catalytic testing, 1-3 mg of Ag NPs or Au NPs were dispersed in 1 ml of MilliQ (MQ) water and sonicated 1 h at 40°C to improve homogenization. All runs were conducted in a 4-5 ml quartz cuvette with 1 cm of length path and under magnetic stirring. First, calculated volumes of MQ water and 4-nitrophenol solution (5 mmol l⁻¹) were mixed. Then, a known volume of freshly prepared NaBH₄ solution (0.3 mol l⁻¹) was added. The reactions were initiated by adding the indicated amount of the NPs dispersion. The final volume reaction was always 3 ml. Once the initial loading of p-NP was almost depleted, an additional pulse of p-NP was injected to the cuvette to assess catalyst stability after the first reaction cycle.
The following operating conditions were analysed: [4-nitrophenol] = 0.1 mmol l\(^{-1}\); [NaBH\(_4\)] = 5; 10; 100 mmol l\(^{-1}\); [NPs] = 8 mg ml\(^{-1}\); 500 rev min\(^{-1}\); 25°C. The reaction progress was periodically monitored through the acquisition of UV-Vis spectra (\(\lambda = 190–1000\) nm) by means of a UV-VIS spectrophotometer (Agilent 8453) with a diode array equipped with a Peltier cell and stirring device. The maximum absorbance wavelength (\(\lambda_{\text{max}}\)) of p-NP is in the visible range, at 400 nm. Outcomes reported in this work represent the mean of 4–5 identical runs.

**PCR reaction in presence of Au NPs**

To analyse the effect of Au NPs on the specificity of PCR reactions three different templates were tested: purified plasmid pRV1 containing a cloned fragment of 1383 bp corresponding to the \(H.\) volcanii hcpG gene (HVO_2150) and \(E.\) coli GM33 or \(H.\) volcanii H26 colonies transformed with the same plasmid. The amplicon size was 1383 bp and the specific primers used were: forward: 5’-AATctcgagGGCCGTTCGTCGGCCT-3’; reverse: 5’-ACggtaccTTAatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgatgcgtacctccgag-3’.

Reaction conditions were as suggested by the supplier (Embiotec, Buenos Aires, Argentina). For the test samples, 1 µl of Au NPs (10 µg ml\(^{-1}\)) was added to the premix (final volume of 25 µl). PCR products were analysed by agarose gel (1.5%, p/v) electrophoresis.

**Results**

**Haloferax volcanii** can synthesize Ag and Au NPs

In order to assess the ability of \(H.\) volcanii to synthesize metallic NPs, cells of this halophilic archaeon were incubated either with AgNO\(_3\) or HAuCl\(_4\) in the presence of metallic NPs, cells of this halophilic archaeon were incubated with HAuCl\(_4\) or AgNO\(_3\) in the presence of NaCl. After the incubation period (1–16 h), the colour of the reaction mixtures changed from cloudy-pink to dark brown, in the case of Ag and from light-orange to purple in the case of Au. These changes are usually a consequence of the formation of metallic particles due to the presence of reducing agents. In samples containing Ag, a first change was observed due to the precipitation of AgCl conferring turbidity to the solution that, in presence of reddish \(H.\) volcanii gave the reaction medium a pink-cloudy aspect. After a 1 h incubation period, the mixture started turning brown, until a dark brown colour was observed in samples incubated for 16 h (ON), a colour change indicative of Ag NPs synthesis (Fig. 1a). When \(H.\) volcanii cells were incubated with HAuCl\(_4\), the initial yellow auri salt solution changed to orange in presence of the micro-organisms and then became a purple-like dispersion as a consequence of the formation of metallic NPs (Fig. 1b). Interestingly, for the metal reduction to occur the active metabolism of the micro-organism was required, since no colour change was observed when boiled (Fig. 1a, b) or sonicated (data not shown) \(H.\) volcanii cells were assayed. UV-Vis absorption spectrum analysis confirmed the presence of nanostructures with surface plasmon resonance bands typical of noble metals. In the samples containing Ag, a maximum absorption peak around 420 nm was observed (Fig. 1c), which is consistent with the presence of Ag nanostructures. In the case of Au, the UV-Vis spectra showed a broad absorption peak around 550 nm (Fig. 1d), coincident with the absorption peak of nanometric Au. The width of the peak suggested size dispersion and possible aggregation of Au NPs. Small variations in peak maximums were observed for both types of NPs in independent synthesis reactions (410–423 nm for Ag and 544–566 nm for Au), which could be a consequence of slight differences in experimental conditions that might affect either the size of the resulting NPs and/or the refraction index of the NPs dispersion.

The amount of metallic salt present in the mixture is probably a limiting factor for NPs synthesis. Therefore, the optimal initial concentration of metallic salt was determined by performing the synthesis reaction at different concentrations. The initial metallic salt concentration that produced highest accumulation of NPs was 1 mmol l\(^{-1}\) for both, Ag and Au (Fig. S1). Higher concentrations of Ag revealed wider peaks (indicating size poly dispersion) whereas for Au, concentrations of 5 mmol l\(^{-1}\) HAuCl\(_4\) or higher resulted to be toxic for the cells, probably due to the acidic nature of HAuCl\(_4\) that in a 5 and 10 mmol l\(^{-1}\) concentration shifted the reaction pH to 5 and 3, respectively.

**Characterization of \(H.\) volcanii** Ag and Au NPs

The elemental composition of the NPs produced by \(H.\) volcanii was determined by means of XRF, confirming the presence of Ag and Au in the analysed material (Fig. S1). The size and shape of the NPs were determined by TEM (Fig. 2) and FESEM (Fig. S2). The synthesized Ag NPs appeared well-dispersed on the TEM grid with average sizes of 25 nm and a spherical-like shape (Fig. 2a, Fig. S2). Au NPs were also spherical with an average size of 10 nm and tended to aggregate forming berry-like bundles (Fig. 2c, Fig. S2). The aggregation of Au NPs was consistent with the wide absorbance peak previously described (Fig. 1d). A total of 184 Ag NPs and 287 Au NPs were measured by means of the Image J software. Ag NPs evidenced a wider size dispersion (15–40 nm) than Au NPs (10–20 nm) (Fig. S2). In order to obtain a clean colloidal suspension of both, Ag and Au NPs, it was necessary to disrupt the cells, suggesting that NPs are produced intracellularly. In addition,
it was also observed that when the culture medium was separated from \textit{H. volcanii} cells, it did not retain the ability to synthesize NPs, indicating that no secreted extracellular molecules were responsible of NP generation (data not shown). TEM images of \textit{H. volcanii} cells after NPs production supported this possibility since NPs were only observed superimposed with the cells (Fig. 2b,d).

NPs synthesized by \textit{H. volcanii} display a nonspecific protein capping

The identification of the biomolecules attached to the NPs was performed by means of FTIR spectroscopy. The infrared spectra of both Ag and Au NPs showed three characteristic bands associated to amide groups arising from proteins. The first one with a maximum at 3279 cm\(^{-1}\), corresponding to N-H stretching and the others located at 1634 and 1534 cm\(^{-1}\), denoted amide I and amide II and assigned to carbonyl stretching and N-H deformation, respectively (Ahsan \textit{et al.}. 2018).

In order to identify the proteins involved in the capping of \textit{H. volcanii} NPs, Ag NPs were treated with SDS at 100°C to release the attached molecules and these proteins were subjected to SDS-PAGE followed by LC/MS-MS. A total of 574 \textit{H. volcanii} proteins were identified (Table S1). The large amount of proteins, the diversity of functional classes and sub-cellular predicted localization suggested that the interaction with the NPs is likely nonspecific.
Ag NPs biosynthesized by H. volcanii show antimicrobial activity

The antimicrobial activity of the Ag NPs synthesized by H. volcanii was tested against two gram-negative bacteria, E. coli and P. putida, by the agar diffusion assay. Inhibition of bacterial growth was observed as a halo with no bacterial growth in the area where Ag NPs were added (Fig. 5), indicating that the Ag NPs synthesized by H. volcanii display antibacterial activity.

Ag and Au NPs can catalyse the p-NP reduction in the presence of NaBH₄

The as-synthesized NPs were evaluated in the reduction of p-NP (0·1 mmol l⁻¹) at 8 mg ml⁻¹ catalyst concentration and with varying NaBH₄ concentrations (5, 10 or 100 mmol l⁻¹). For NaBH₄ concentrations up to 10 mmol l⁻¹, no activity was observed within, at least, 24 h, suggesting limited access of reagents to the active sites of NPs probably as a consequence of the proteins that stabilize the metal NPs (Fig. 6, grey circles). However, the reaction proceeded by using 100 mmol l⁻¹ of NaBH₄ (Fig. 6, black circles) conditions at which the catalytic performance of the biosynthesized NPs was significantly improved. This change was probably due to an enhancement of the accessibility of reactants to the active surface. Figure 5 illustrates the performance of Au and Ag NPs in two consecutive cycles of use. The induction period observed in the first cycle was 2 ± 0·2 and 4 ± 0·6 min with Au and Ag NPs, respectively. In both cases, the initial p-NP was reduced in <25 min. In a second cycle, after a subsequent addition of p-NP, the induction period was no longer observed whereas both catalysts were capable of effectively reducing p-NP.

Au NPs synthesized by H. volcanii improve the specificity of a PCR reaction

The effect of the H. volcanii biosynthesized Au NPs on a standard PCR reaction was tested. The Au NPs were used directly without any pretreatment, (i.e. with an intact
capping protein shell). The PCR reaction was carried out using three different DNA templates (purified plasmid, bacterial and archaeal colonies). It was clearly observed that the nonspecific tailing bands fainted when Au NPs were added to the reaction mix, indicating that the Au NPs produced by *H. volcanii* could increase the specificity of the PCR reaction (Fig. 7).

**Discussion**

The utilization of organisms of the Archaea domain for NPs synthesis is not widespread, even though the extreme conditions which these organisms often require are accompanied by physiological unique traits that could be exploited for biotechnological processes. In this work, the biosynthesis of Ag and Au NPs by the model haloarchaeon *H. volcanii* was demonstrated for the first time. The use of this micro-organism allowed the production of Au and Ag NPs through a fast and easy procedure. Haloarchaea grow in environments containing from 1.5 mol l⁻¹ to saturating NaCl concentrations which are conditions particularly useful for avoiding culture contaminations with mesophilic micro-organisms in biotechnological processes. Both Ag and Au NPs were synthesized intracellularly. Extracellular NPs synthesis is usually preferred for industrial applications as intracellular synthesis requires the internalization of the metal ions (Hulkoti and Taranath 2014), which may slow NPs generation, and complex/expensive downstream treatments in order to obtain the colloidal NPs suspension. However, even though NPs biosynthesis by living micro-organisms usually requires 24–120 h (de Souza *et al*. 2019), *H. volcanii* seems to be faster as an evident colour change was already observed after 1 h of incubation, effect that increased until reaching a maximum (verified as top intensity of the plasmon band) after 16 h (Fig. 2). In addition, the possibility of disrupting cells by osmotic shock (only adding water) avoids the application of expensive specialized equipment to obtain free NPs.

![Figure 3](https://example.com/figure3.png)

**Figure 3** Transmission electron microscopy of NPs synthesized by *Haloferax volcanii*. Micrographs a and b correspond to purified samples of Ag and Au NPs, whereas c and d were taken from whole *H. volcanii* cells used during synthesis of Ag and Au respectively. Insets show magnified images of individual NPs. Arrows point to the place in which some NPs appeared superimposed to *H. volcanii* cells. [Colour figure can be viewed at wileyonlinelibrary.com]
*Haloferax volcanii* cells needed to be alive in order to synthesize NPs, as boiled (Fig. 1) or sonicated cells were not able to produce Ag or Au NPs. The optimal metal salt concentration for *H. volcanii* NP biosynthesis was 1 mmol l\(^{-1}\) (Fig. 2). No Au NPs were produced at HAuCl\(_4\) concentrations of 5 mmol l\(^{-1}\) or higher, reactions in which the pH decreased to values between 3 and 5. *Haloferax volcanii* grows optimally at pH 7-4 and no growth in liquid medium is observed when pH is below 5.5 (Moran-Reyna and Coker 2014), therefore, it is likely that the cells died when they were incubated with 5–10 mmol l\(^{-1}\) HAuCl\(_4\) and, in consequence, were not able to produce Au NPs.

The NPs synthesized by *H. volcanii* were characterized using TEM (Fig. 3) and FESEM (Fig. S2) to determine shape and size, XRF to confirm the presence of Ag and Au in the obtained material (Fig. S1) and FTIR to identify surface-associated molecules (Fig. 4). Electronic microscopy observations evidenced spherical NPs with average sizes of about 25 nm (Ag) and 10 nm (Au). When cells were not disrupted prior to TEM observation, NPs were detected superimposed with *H. volcanii* (Fig. 3). As TEM provides 2D images, these results are not a proof of the presence of NPs inside the cell but strongly support this hypothesis. NPs capping with a protein shell is an advantage as it increases the solubility and colloidal stability in an aqueous environment (Lin-Vienet et al. 1991). In this work, we showed that *H. volcanii* synthesized Ag and Au NPs display a protein capping by means of a FTIR analysis (Fig. 4). *Haloferax volcanii* has a high proportion of proteins (almost 90% of the predicted proteome) which are negatively charged at pH 7-4 (the optimal pH for growth). Hence, electrostatic interactions could account for the efficient stabilization of *H. volcanii* NPs in the aqueous dispersion. In order to go deeper into the protein corona composition, a sample of Ag NPs was treated with SDS and heated to release the attached proteins. The released material was analysed by LC-MS/MS and 574 proteins were identified. In many bacteria the synthesis of Ag NPs relies in the occurrence of enzymes with oxidoreductase activity (redox enzymes) such as nitrate reductase in *Pseudomonas aureginosa* (Ali et al. 2017) and *E. coli* (Shahverdi et al. 2007) or C-type cytochromes in *Shewanella oneidensis* (Huang et al. 2015) and *Desulfovibrio desulfuricans* ATCC 29577 (Mabbett et al. 2004). In archaea, the involvement of nitrate reductase has been reported in the biosynthesis of Ag NPs by *Halococcus salifodinae* BK3 (Das et al. 2014). Although in this work only one sample was analysed by LC/MS-MS, it is interesting to note that 36 reductases were detected in association with Ag NPs (corresponding to 6-2% of the total of proteins identified in this assay vs 3-3% predicted reductases in the complete *H. volcanii* proteome). This result could be a preliminary indicator of the involvement of reductase enzymes in Ag NPs synthesis in *H. volcanii*. Further work will be necessary for elucidation of the reduction mechanism.

The resistance of bacteria to conventional antibiotics is a public health issue which highlights the need for the development of novel antibacterial compounds. One of the alternatives to conventional antibiotics is the utilization of Ag NPs which are currently used in several commercial products and in medicine (Bondarenko et al. 2018). The Ag NPs produced by *H. volcanii* displayed antibacterial activity against *E. coli* and *P. putida* (Fig. 5) and therefore constitute promising candidates as antimicrobial agents. A similar observation was made for AgNPs synthesized by *H. salifodinae* BK3 (Srivastava et al. 2013). The mechanisms underlying metal NPs toxicity on micro-organisms are not completely understood.

![Figure 4](image_url) FTIR spectrum of nanoparticles (NPs) synthesized by *Haloferax volcanii*. Ag (a) and Au (b) NPs showed three characteristic bands associated to proteins: 3279 cm\(^{-1}\) (Amide A), 1634 cm\(^{-1}\) (Amide I) and 1534 cm\(^{-1}\) (Amide II). [Colour figure can be viewed at wileyonlinelibrary.com]
Recently, it was proposed that Ag NPs target the inner membrane of Gram-negative bacteria producing depolarization which in turn inhibits cellular respiration (Djurisic et al. 2015).

The reduction of p-nitrophenol (p-NP) in the presence of NaBH₄ is frequently selected as the model reaction for assessing the catalytic performance of metal NPs (Zhao et al. 2015). The reaction generally proceeds after an

Figure 5 Antibacterial activity of Ag NPs. A plate diffusion assay was used to determine inhibition of Escherichia coli (a) and Pseudomonas putida (b) growth in LB-agar. Ampicillin (Amp) was used as a positive inhibition control and sterile bi-distilled H₂O as a negative control. The inhibition halos were measured after 16 h incubation at the optimal growth temperature of each micro-organism. Bar plots are representative of nine independent experiments and statistically significant differences were determined at \( P < 0.05 \). An example picture of the observed halos is included at the bottom of each graph. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 6 Temporal evolution of normalized 4-nitrophenol (4-NP) concentration. (a) as-synthesized Au NPs and (b) as-synthesized Ag NPs in two consecutive reaction experiments using (○) \([\text{NaBH}_4]\) = 10 mmol l⁻¹; (●) initial \([\text{NaBH}_4]\) = 100 mmol l⁻¹. Other conditions: 25°C, 500 rev min⁻¹, [4-NP] = 0.1 mmol l⁻¹, [NPs] = 8 μg ml⁻¹.

(Bondarenko et al. 2018). Recently, it was proposed that Ag NPs target the inner membrane of Gram-negative bacteria producing depolarization which in turn inhibits cellular respiration (Djurisic et al. 2015).
induction period towards the formation of p-aminophenol. The sequence of reagents addition, the operating conditions as well as the nature of the capping ligands have shown to strongly affect both the induction period and p-NP reduction rate (Álvarez-Cerimedo et al. 2019). *Haloferax volcanii* Ag and Au NPs were able to catalyse p-NP reduction in presence of 100 mmol l⁻¹ NaBH₄ (Fig. 6). This is in line with previous works reporting that the addition of a high concentration of NaBH₄ promoted the surface replacement of ligands (e.g. thiols or PVP) by borohydride or hydride anions (Ansar et al. 2013; Ansar and Kitchens 2016; Álvarez-Cerimedo et al. 2019), enhancing accessibility of the reactants to the NPs surface. In this case, partial replacement of proteins by these anions may explain the observed catalytic improvement.

The inclusion of Au NPs in a PCR mix may improve the specificity and yield of PCR reactions, by reducing base mispairing (Li et al. 2005; Girilal et al. 2013). This fact can be explained by the remarkable heat transfer properties of the Au NPs in aqueous solution that enhances the chemical reaction, besides their higher affinity towards single-stranded DNA rather than to double stranded DNA. In this work we showed that *H. volcanii* synthesized Au NPs evidenced the ability of increasing the specificity of a PCR reaction, as addition of as-synthesized Au NPs resulted in fainting of nonspecific PCR products (Fig. 7).

In conclusion, we showed that the archaeon *H. volcanii* can be used to biosynthesize Ag and Au NPs by an easy, fast and low-cost procedure. The material obtained was characterized and three possible applications (antibacterial, PCR specificity enhancer and catalytic activities) were explored. Taken together, the results presented in this work provide the fundamental base for future studies that may help to understand the physiological significance of NP synthesis in halobacteria and for the development of technological applications of NPs produced by *H. volcanii*.

**Acknowledgements**

This work was supported by the National Agency of Scientific and Technological Promotion (PICT1477/14, awarded to R.E.D. and PICT1433/15 awarded to C.E.H.) and UNMdP (EXA823/17, awarded to R.D.C. and M.I.G.). The authors acknowledge Vanesa M. Fuchs (INTEMA–CONICET–UNMdP) for assistance with the XRF analysis. M.I.C. is a PhD fellow sponsored by the CONICET (Argentina).

**Conflict of Interest**

No conflict of interest declared.

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**Figure 7** Effect of Au NPs in PCR reaction specificity. Au NPs (10 ng) were added either in presence or absence of Au NPs using three different templates: purified plasmid (a), *Escherichia coli* colony transformed with the same plasmid (b) and *Haloferax volcanii* H26 colony containing the same construction. Amplicons were observed in agarose gels stained with SybrSafe. [Colour figure can be viewed at wileyonlinelibrary.com]
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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Figure S1. X-ray fluorescence (XRF) analysis of NPs synthesized by *H. volcanii*.

Figure S2. Field emission scanning electron microscopy of NPs synthesized by *H. volcanii*.

Table S1. Ag NPs associated proteins identified by LC-MS/MS.