Antimicrobial activity of biosilver nanoparticles produced by a novel Streptacidiphilus durhamensis strain

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Abstract Background/Purpose: In this study, an acidophilic actinobacteria strain was used as a novel reducing agent for a single-step synthesis of nanostructure silver particles. We used a Streptacidiphilus durhamensis HGG16n isolate for efficient synthesis of bioactive silver nanoparticles [bio(AgNPs)] in an inexpensive, eco-friendly, and nontoxic manner. The obtained bio(AgNPs) exhibited unique physicochemical and biochemical properties.

Methods: Structural, morphological, and optical properties of the synthesized biocolloids were characterized by spectroscopy, dynamic light scattering, and electron microscopy approaches. The antimicrobial activity was evaluated using the well- and disc-diffusion methods.

Results: The obtained crystalline structure and stable biosynthesized silver nanoparticles ranged in size from 8 nm to 48 nm and were mostly spherical in shape. Antimicrobial assays of the silver nanoparticles against pathogenic bacteria showed the highest antimicrobial activity against Pseudomonas aeruginosa, Staphylococcus aureus, and Proteus mirabilis, followed by Escherichia coli, Klebsiella pneumoniae, and Bacillus subtilis. Moreover, the synergistic effect of bio(AgNPs) with various commercially available antibiotics was also evaluated.

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Introduction

Development of biologically inspired experimental processes involved in the synthesis of silver nanoparticles (AgNPs) is evolving into an important branch of nanotechnology. Physical and chemical techniques tend to be capital-intensive, inefficient in materials and energy use, and may require toxic or environmentally harmful chemicals. Nanoparticle synthesis involving the use of biological systems might offer inexpensive, clean, non-toxic, and eco-friendly alternatives. Biosynthesis is a type of a bottom up approach where formation of nanoparticles occurs due to reduction/oxidation of a metal, and the agents mainly responsible for these processes are different enzymes secreted by microbial systems.1,2

Green synthesis of AgNPs using bacteria has been shown in fungi, algae, and plants.3–6 However, among the different microorganisms applied to the synthesis of AgNPs, actinobacteria are less known.7 Previous studies showed that nanoparticles synthesized by individual members of actinobacteria presented good polydispersity and stability and possessed significant biocidal activity against various pathogenic microorganisms.8–9 However, little is known about metal-nanoparticle synthesis by actinobacteria isolated from acidic environments. Acidic forest soil is an extreme environment for many microorganisms (pH < 4.0); however, it contains a large number of acid-loving actinobacteria that produce antimicrobial compounds10 with unknown biological activity.

Applications in the field of medicine include formulations of many potential antimicrobial agents that are effective against human pathogens, including multidrug-resistant bacteria.11 Multi-drug resistant strains of bacteria have become a serious public health problem.12 The emerging resistance of bacteria and the high cost of advanced antimicrobial drugs have encouraged scientists to search for effective, economically viable, and broadly applicable drugs.13 Therefore, development of novel antimicrobial compounds or modification of the available ones to combat resistant pathogens is urgently needed. AgNPs produced by microorganisms are good candidates for a new generation of antimicrobial materials.14

This study focused on biogenic synthesis of metal nanoparticles by acidophilic actinobacteria strain HGG16n and evaluation of their antimicrobial activity. Physicochemical characteristics of the biosynthesized AgNPs was also determined.

Conclusion: These results provide insight into the development of new antimicrobial agents along with synergistic enhancement of the antibacterial mechanism against clinical bacteria. Copyright © 2017, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Methods

Synthesis of bio(AgNPs) by HGG16n isolate

For synthesis of the AgNPs, 100 mL yeast malt agar (ISP2) broth was prepared in a flask, sterilized, and inoculated with a fresh culture of HGG16n strain. The inoculated flasks were incubated in an orbital shaker (150 rev min⁻¹) at 26°C for 14 days, followed by centrifugation at 10414 g for 15 minutes. The supernatant was then combined with 3mM AgNO₃ (1:1, v/v) and incubated at 26°C in a shaker for 7 days in the dark. The synthesis of bio(AgNPs) was observed by the color change from yellow to dark brown. ISP2 broth mixed with 3mM AgNO₃ (1:1, v/v) was used as a control. Aliquots were collected at different time intervals and analyzed by UV–visible spectra indicated (Vis) spectroscopy (NanoDrop ND2000, Thermo Scientific, Carlsbad, CA, USA). The λ_max was measured within the range of 200–800 nm.

Removal of unreacted Ag ions and sample purification

Nanoparticles were centrifuged at 25200 g for 30 minutes to concentrate, then resuspended three-fold in deionized water and centrifuged at 12857 g. To remove the unreacted Ag⁺ and low molecular weight metabolites from the native nanoparticle sample, a combination of a 3-day dialysis (3-kDa cut-off; Spectrum Laboratories, Houston, TX, USA) and addition of a 0.9% NaCl solution as a control was performed. Bio(AgNPs) were also dried at 50°C and stored at −20°C.

Characteristics of bio(AgNPs)

Elemental analysis and inductively coupled plasma mass spectrometry

Samples were suspended in 0.87% KCl, then vortexed for 5 minutes and sonicated in an ultrasonic bath for 20 minutes. Elemental composition was evaluated using an elemental analyzer (Vario MACRO CN; Elementar Analysensysteme GmbH, Hanau, Germany). Samples of AgNPs were dissolved in 15% (v/v) nitric acid and then diluted to 1% (v/v). Ag concentration was estimated with the use of a CX 7500 inductively coupled plasma mass spectrometry (ICP-MS) spectrometer (Agilent, Santa Clara, CA, USA).

Fourier-transmission infrared spectroscopy

Characterization of functional groups on the surface of bio(AgNPs) was investigated using a Fourier-transmission infrared
spectrum (FT-IR) Spectrum 2000 spectrometer from Perkin-Elmer (Waltham, MA, USA). A sample was prepared by uniform dispersion of bio(AgNPs) in a dry KBr matrix and compression to form a disc. The FT-IR spectrum was recorded in the range of 4000—500/cm. The obtained data was evaluated with WINFIRST software (Mattson, Fremont, CA, USA).

**Dynamic light scattering and zeta potential**

The size and zeta potential of bio(AgNPs) were analyzed by Zetasizer Nano Series (Malvern Instruments, Malvern, UK). Size distribution analysis was performed using the dynamic light scattering (DLS) method. Immediately before measurement, sample was suspended in 0.87% KCl, vortexed for 5 minutes, and sonicated in an ultrasonic bath for 20 minutes. All measurements were performed in triplicate for each sample using UV-grade cuvettes for size determination and folded capillary cells for registering the zeta potential (ZP).

**Electron microscopy**

Distribution of bio(AgNPs) size was investigated by scanning electron microscopy (LEO 1430VP; Carl Zeiss, Oberkochen, Germany) coupled with an energy-dispersive X-ray (EDX) detector (XFlash 4010; Bruker, Billerica, MA, USA) and transmission electron microscopy (TEM; FEI Tecnai F20 X-Twin; FEI, Hillsboro, OR, USA). A sample was dropped onto a carbon-coated copper grid and the excess solution was removed. The obtained data was evaluated by Statistica Software (StatSoft Inc., Tulsa, OK, USA) using the variability plot of average methods.

**Tested organisms**

The Gram-positive *Staphylococcus aureus* ATCC6338, *Bacillus subtilis* PCM2021, and Gram-negative *Escherichia coli* ATCC8739, *Pseudomonas aeruginosa* ATCC10145, *Klebsiella pneumoniae* ATCC700603, *Proteus mirabilis* (from the collection of the Collegium Medicum of Nicolaus Copernicus University, Torun, Poland), and *Salmonella infantis* (from the Sanitary-Epidemiological Station in Torun, Poland) bacterial pathogens were tested.

**Antimicrobial assay of bio(AgNPs) and their synergistic effect combined with commercial antibiotics**

Seven clinical isolates were screened for their sensitivity to the synthesized AgNPs and standard antibiotics (Thermo Fisher Scientific Oxoid, Ltd., Basingstoke, UK), including streptomycin (25 mcg/disc), gentamycin (30 mcg/disc), kanamycin (5 mcg/disc), ampicillin (25 mcg/disc), tetracycline (30 mcg/disc), and neomycin (30 mcg/disc) by the well- and disk-diffusion methods. Samples (AgNPs and standard antibiotic discs) were aseptically placed on tryptic soy agar (TSA) plates inoculated with 100 μL of 1 × 10⁸ bacterial suspension. Plates were subsequently incubated at 37°C for 24 hours, followed by observation of inhibition zones. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of bio(AgNPs) was determined using the microtiter broth-dilution method. All determinations were performed in triplicate.¹⁵

**Atomic-force microscopy analyses of bacterial cells after bio(AgNP) treatment**

Ten microliters of a sample containing bacterial cells in tryptic soy buffer (TSB; 1 × 10⁶ colony forming units/mL) combined with the biosynthesized AgNPs (100 μg/mL) were applied on a gelatin-coated glass surface.¹⁶ The samples were incubated at room temperature for 20 minutes, then gently rinsed with sterile deionized water and nitrogen-dried before imaging by atomic-force microscopy (AFM). Bacterial cells in TSB were used as a control.

Alterations in the bacterial cell surface caused by AgNPs were imaged in tapping mode in air using metal-to-ligand charge transfer (MLCT) silicon nitride cantilevers with a resonant frequency of 7 kHz. AFM was carried out with a Veeco Bioscope II atomic-force microscope (Veeco, Santa Barbara, CA, USA). The scan rate and resolution of the obtained scans were 0.5 Hz and 512 pixels × 512 pixels, respectively. Topography and deflection images were obtained simultaneously. The obtained data were analyzed with Gwyddion 2.41 software (http://gwyddion.net/).

**Results**

**Identification of HGG16n isolate**

A nearly complete 16S rRNA gene sequence (1387 nucleotides) of HGG16n isolate was determined. The isolate was most closely related to *Streptacidiphilus durhamensis* FSCA67T and shared 16S rRNA similarity with the above-mentioned strain, corresponding to a difference in 1387 nucleotide locations.¹⁷,¹⁸

**AgNP biosynthesis**

UV-Vis biosynthesis of AgNPs by *Streptacidiphilus durhamensis* in cell-free extracts after addition of 3mM AgNO₃ solution. A lack of color change was observed in the control, which contained medium with a solution of AgNO₃ (Figure 1A). The presence of bio(AgNPs) was also confirmed by elemental analysis (EA) and ICP-MS.

**EA and ICP-MS**

EA showed that the obtained bio(AgNPs) contained 25.73 ± 0.01% of carbon, 5.89 ± 0.04% of hydrogen, and 3.47 ± 0.02% of nitrogen. The content of Ag was estimated by ICP-MS, and the concentration of bio(AgNPs) in the culture medium was 10.40 ± 0.01 mg/L.

**FT-IR**

The FT-IR spectrum (Figure 2) of HGG16n AgNPs showed changes in both the form and the peak position in the region of 4000—500/cm. KBr spectra in the analyzed range (4000—500/cm) showed insignificant signals not affecting the spectrum of AgNPs. Vibration bands observed in this region (3427.7/cm, 3437.9/cm, and 3421/cm) are characteristic for vibrations of hydroxyl and amino groups.¹⁹ Another common characteristic band at ~1384.4/cm
(Figures 2B and 2C) was assigned to N—O-stretching vibration.\textsuperscript{19} In the case of AgNPs, this signal was less intensive as compared with the AgNO\textsubscript{3} band, and could be a result of amine oxidation localized onto biocolloid surfaces. A new signal at 1623/cm that was not observed in the A or B spectra could be a result of amide I vibration, which originates mainly from stretching of carbonyl groups (C=O). A band near 1480/cm was also new and assigned to C—N stretching and NH bending,\textsuperscript{15} indicating the presence of aliphatic groups of amide II. Another new band that contributed to C=O vibrations of carboxylic groups was also previously reported at 1123/cm.\textsuperscript{19} FT-IR results indicated the presence of proteins or peptides on the surface of the synthesized AgNPs.\textsuperscript{15} The signals obtained at 800—875/cm showed the presence of absorption bands stemming from C—Br vibration, which should correspond to the used pellets.\textsuperscript{19}

**DLS and ZP**

The hydrodynamic size of solvated bio(AgNPs) was found to be smaller than 100 ± 1.5 nm (Figure 3A). The size of the nanoparticles decreased from 700 nm to 200 nm at pH ranges of 2—4. Nanoparticles with sizes < 100 nm were obtained in the pH range of 5—10. At pH > 10, an increase in bio(AgNPs) size was observed, resulting from system destabilization, denaturation of organic compounds localized onto biocolloid surfaces, and oxidation and/or hydroxyoxidation of Ag.

According to Figure 3B, the maximum ZP value was approximately −32 mV, which indicated that the synthesized particles were stable due to electrostatic repulsion between the solvated particles.\textsuperscript{20} At pH 2—4, the ZP value of bio(AgNPs) was 0 ± 0.5 mV. In this pH range, the system was unstable and aggregated. At pH 5—10, ZP oscillation was observed, and at pH > 10, we registered a plateau of the model curve. Size measurements demonstrated creation of a hydroxyl complex of Ag and degradation of its organic parts. Consequently, a correlation between the size distribution and the ZP of bio(AgNPs) analyzed at different pH values was observed.

**EM of bio(AgNPs)**

Metallic Ag was noticed on the grid surface (Figure 4A). The synthesized bio(AgNPs) had spherical shape (Figure 4B), and the distance between the two atomic layers was 0.24 nm, which is characteristic of atomic layers of Ag (Figure 4C).\textsuperscript{21} Highly intense signals revealed the presence of elemental Ag. EDX spectra of bio(AgNPs) (Figure 4D) confirmed that Ag was the major element, with a ~3-keV signal.\textsuperscript{22} The detected minor amounts of carbon and oxygen could be assigned to organic compounds attached to the Ag core, and the presented copper signals could be corresponded to the TEM grid. Figure 4E illustrates the percentage of the
particle-size distribution. Heterogeneous size distribution was observed in the range of ~8–48 nm with the following values: 28% (10–30 nm), 24% (15–20 nm), 21% (13–18 nm), 16% (8–40 nm), 8% (9–48 nm), and 3% (28 nm). The average value was 23 ± 2 nm.

**MIC and MBC of bio(AgNPs)**

Bacteriostatic activity of bio(AgNPs) toward *B. subtilis*, *K. pneumoniae*, *E. coli*, *S. aureus*, and *S. infantis* was recorded at low concentration (6.25 µg/mL); however, for *P. mirabilis* and *P. aeruginosa*, this value reached 50 µg/mL and 25 µg/mL, respectively. For *E. coli* and *P. aeruginosa*, the AgNPs exhibited the lowest MBC at 50 µg/mL. Bactericidal activity of bio(AgNPs) for *K. pneumoniae*, *P. mirabilis*, *B. subtilis*, and *S. aureus* was 100 µg/mL (Table 1).

**Antimicrobial activity of bio(AgNPs)**

The well-diffusion method showed the highest antimicrobial activity against *P. aeruginosa*, *S. aureus*, and *P. mirabilis* (10 mm for all), followed by *E. coli*, *K. pneumoniae*, and *B. subtilis* (6 mm for all). No bio(AgNPs) activity was detected against *S. infantis*. We also observed synergistic effects of bio(AgNPs) combined with antibiotics against bacterial pathogens (Table 1). Increased antibacterial activities of kanamycin, ampicillin, and tetracycline in the presence of bio(AgNPs) against *K. pneumoniae*, *S. aureus*, and *P. aeruginosa* were observed, while *P. mirabilis* was the most resistant bacterium to the applied antibiotics. However, an increase in streptomycin activity against *P. mirabilis* was observed when it was combined with bio(AgNPs). Synergistic effects

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**Figure 3.** Size of the bio(AgNPs) and their activity according to pH value. (A) Zeta potential of bio(AgNPs) according to pH value. (B) Error limits (red shadow). bio(AgNP) = bioactive silver particles.
with the remaining antibiotics to this Gram-negative bacterium were not observed. AFM imaging of bio(AgNPs) activity on *B. subtilis*, *E. coli*, and *S. aureus* is presented in Figure 5.

**Discussion**

The formation of Ag nanoparticles can be primarily confirmed through observation of the change in solution color in visible light (Figure 1B). Brown color is the result of excitation of surface plasmon vibration typical for Ag nanoparticles. A peak close to 420 nm associated with the surface plasmon resonance is well-documented for various metal nanoparticles with sizes ranging from 2 nm to 100 nm.15,24

**DLS and ZP**

The absolute ZP of a biological system at ±35 mV suggests stable dispersion.25 Unstable aggregates created in a pH range of 2–4 can be correlated with functional-group deprotonation (mostly carboxyl groups) in the organic part of bio(AgNPs). This phenomenon is caused by the occurrence of weak repulsion and strong attractive forces.
between solvated particles.\textsuperscript{25} In our case, ZP oscillation at pH 5–10 was caused by the following deprotonation and/or protonation of the functional groups present in the bio-nanocomplex. Deprotonation results from increased negative charge and increases stability of the nanoparticles. Moreover, enhanced repulsive forces cause an increase in dispersion of bio(AgNPs).

The electron microscopy study of bio(AgNPs)

The larger bio(AgNPs) observed by DLS (100 nm) as compared to TEM analysis (23 nm) resulted from the different measurement approaches. The obtained AgNPs size according to DLS can be influenced by a particle core, the possibility of aggregation, branching of the organic part, and consequently its solvation.\textsuperscript{26,27} FT-IR confirmed the presence of the organic part. It is possible that proteins could be involved in reduction of Ag\textsuperscript{+} ions into Ag.\textsuperscript{2} Proteins are able to form bonds with AgNPs through free amino groups present in the protein structures.\textsuperscript{28} Moreover, the stretching vibration of carbonyl amino groups recognized as amide I and amide II was observed, and appeared to be due to amide linkages with a protein, which is commonly responsible for the reduction process. The presence of hydroxyl and carbonyl groups on the AgNPs indicated extracellular mechanisms associated with Ag reduction. The presence of carbonyl, amino, and hydroxyl groups in the system can indicate protein-layer formation onto AgNPs, affecting nanoparticle stabilization in culture media.\textsuperscript{29} A previous study confirmed the presence of organic sheaths on the surface of AgNPs was total organic carbon concentration of 0.89 mg/mL organic carbon per 1 mg of Ag.

**Antimicrobial activity of bio(AgNPs)**

The enhanced ability of bio(AgNPs) to inhibit the growth of \textit{P. aeruginosa}, \textit{S. aureus}, and \textit{P. mirabilis} confirmed that the nanoparticles obtained from the actinobacteria HGG16n strain had great potential to be used as antimicrobial agents against pathogenic microorganisms. We observed that the antimicrobial proficiency of the AgNPs found against \textit{E. coli} and methicillin-resistant \textit{S. aureus}.\textsuperscript{29} The absence of antibacterial activity on the part of the AgNPs was also confirmed against \textit{E. coli} and \textit{S. infantis} could be associated with the method used, as well as the ability of \textit{Salmonella} spp. to form biofilms.\textsuperscript{30} This phenomenon might lead to precipitation and/or limited diffusion of AgNPs in TSA as a result of clogging of the gel pores. Furthermore, the same strain of bacteria examined in TSB medium to determine MIC and MBC presented high antimicrobial effects, in contrast with results from the well-diffusion method.

AFM imaging showed that the bio(AgNPs) from thHGG16n strain caused damage to bacterial cells, including \textit{B. subtilis}, \textit{E. coli}, and \textit{S. aureus}, as compared to the controls (Figure 5). AFM was previously used for AgNP characterization;\textsuperscript{31} however, results associated with bacterial cell analyses following treatment with biosynthesized nanoparticles using AFM are relatively new.

### Table 1 Antimicrobial activity of bio(AgNPs) from HGG16n strain against bacterial pathogens.

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>Tested organisms</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
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<tr>
<td></td>
<td>Escherichia coli ATCC8739</td>
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<tr>
<td></td>
<td>Salmonella infantis ATCC700603</td>
<td>6.25</td>
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<tr>
<td></td>
<td>Klebsiella pneumoniae ATCC23499</td>
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<td></td>
<td>Pseudomonas aeruginosa ATCC10145</td>
<td>6.25</td>
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<td></td>
<td>Proteus mirabilis</td>
<td>6.25</td>
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</table>

\(A\) = ampicillin; \(b\) = bioactive silver nanoparticles; \(G\) = gentamycin; \(IZ\) = inhibition zones in diameter (mm); \(K\) = kanamycin; \(MBC\) = minimum bactericidal concentration; \(MIC\) = minimum inhibition concentration; \(N\) = neomycin; \(NI\) = no inhibition; \(S\) = streptomycin; \(SD\) = standard deviation; \(T\) = tetracycline.

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The antibacterial activity of AgNPs results from their penetration into a bacterium, attachment to the surface of a cell membrane, and disturbance of energy production by damage to the cell membrane, followed by the release of cell contents. AgNPs attack Gram-negative bacteria by anchoring and penetrating the cell wall, leading to structural changes in the cell membrane that increase permeability. The antibacterial mechanisms associated with AgNPs are attributable to the formation of free radicals that induce membrane damage. Ag ions interact strongly with thiol groups in enzymes and phosphorus-containing bases, and AgNP interaction with DNA may prevent bacterial cell division and DNA replication, leading to cell death.

Combinations of antibiotic and AgNP treatment increased bactericidal efficiency against bacterial cells.
relative to their separate application.17,18,32–36 This synergism was likely the result of binding of the antibiotic molecules to AgNP functional groups, including carboxyls, resulting in their operation as antibiotic carriers.

The antibacterial activity of nanoparticles is determined by their size, shape, and concentration. Large nanoparticles allow large surface areas to contact bacterial cells, meaning that smaller particles will potentially have higher percentages of interactions relative to larger particles. Nanoparticles of 25 nm possess the highest antibacterial activity.37,38 Smaller nanoparticles exhibit higher toxicity toward bacterial pathogens, as these nanoparticles likely diffuse more easily relative to those larger in size.8 The antibacterial efficacy of nanoparticles is also influenced by their shape.14 Triangular nanoparticles exhibited almost complete inhibition of bacterial growth at a total silver content of 1 µg, whereas spherical AgNPs needed 12.5 µg, and rod-shaped AgNPs required 50–100 µg of total silver content to significantly reduce colony numbers.39 AgNP biosynthesis is mainly extracellular, with the resulting nanoparticles in the size range of 5–100 nm (mainly 10–50 nm) with a spherical shape.9,37 Our study showed that bio(AgNPs) synthesized by S. durhamensis HGG16n were spherical in shape and in the size range of 8–48 nm (23 ± 2 nm on average).

Our results indicated that this isolate can be used for efficient synthesis of bioactive AgNPs using an inexpensive, eco-friendly, and non-toxic method. Furthermore, the obtained bio(AgNPs) exhibited unique physicochemical and biochemical properties. Our findings provide insight into development of new antimicrobial agents with synergistic enhancement of the antibacterial mechanism against clinical bacteria.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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