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EXPERIMENTAL ARTICLES

Biosynthesis of Gold Nanoparticles by *Azospirillum brasilense*

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Abstract—Plant-associated nitrogen-fixing soil bacteria *Azospirillum brasilense* were shown to reduce the gold of chloroauric acid to elemental gold, resulting in formation of gold nanoparticles. Extracellular phenol-oxidizing enzymes (laccases and Mn peroxidases) were shown to participate in reduction of Au^{+3} ($HAuCl_4$) to Au^0. Transmission electron microscopy revealed accumulation of colloidal gold nanoparticles of diverse shape in the culture liquid of *A. brasilense* strains Sp245 and Sp7. The size of the electron-dense nanospheres was 5 to 50 nm, and the size of nanoprisms varied from 5 to 300 nm. The tentative mechanism responsible for formation of gold nanoparticles is discussed.

Keywords: *Azospirillum*, formation of gold nanoparticles, laccase, Mn peroxidase

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Colloidal gold is presently extensively used in various areas of medicine, biochemistry, immunology, and technology [1]. One of the most intensively developed directions in the field of nanotechnology is studying the properties of gold nanoparticles and the development of techniques for their preparation. The widely used methods apply chemical reduction of gold by reducing agents. However, despite successful application of such methods, they often remain expensive and require environmentally hazardous chemicals. Biological, or so-called green synthesis, including the techniques involving microbial biotechnologies, are therefore of increasing interest [2].

Researchers noted the capability of bacteria for reduction of gold from gold-containing compounds to elementary state, generating gold nanoparticles. Formation of these particles may occur both extracellularly (*Lactobacillus* sp., *Rhodopseudomonas capsulata,* and *Pseudomonas aeruginosa* [3–5]) and inside the cells (*Shewanella algae* and *Stenotrophomonas maltophilia* [6, 7]). Nothing is known about gold reduction by soil bacteria of the genus *Azospirillum,* nitrogen-fixing microorganisms which may form associations with plants and stimulate plant growth and development. The enzymes involved in gold bioreduction to the elementary state are not known. Relatively recently, probable involvement of phenol oxidases in the process has been reported [8]. No experimental data are presently available on gold nanoparticle formation under the effect of homogeneous enzymes.

These considerations determined the goal of this study: to explore the capacity of *A. brasilense* for reduction of gold to the elementary state with the formation of nanoparticles and to determine the role of laccases and Mn peroxidases in this process.

MATERIALS AND METHODS

Organisms and cultivation conditions. Two strains from the collection of microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, *A. brasilense* Sp245 and *A. brasilense* Sp7, were used in this study. Bacteria were cultured at 37°C in a liquid malate–salt medium containing the following (g/L): KH_2PO_4, 0.1; K_2HPO_4, 0.4; NaCl, 0.1; $NaMoO_4 \cdot 7H_2O$, 0.002; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.02; malic acid, 5; NaOH, 1.7; NH_4Cl, 1; $CaCl_2$, 0.02; and $MnSO_4 \cdot 5H_2O$, 0.02; pH 6.8 [9]. A 12-h culture grown in the same medium was used as inoculum. The starting density of the cultures in all experiments was 2×10^7 cells/mL. After 24 h cultivation, an aqueous solution of $HAuCl_4$ (Sigma-Aldrich, United States) at concentrations of 5–500 μM was added to the medium under aseptic conditions.

The effect of chloroauric acid on bacterial growth, as well as the minimal growth inhibiting and minimal lethal concentrations, were determined by changes in the optical density of bacterial suspensions after 18 h of cultivation with $HAuCl_4$ as compared to the control. To determine the number of viable cells, the standard method of CFU count was used.

Enzyme activity determination. Activity of Mn peroxidase was determined by spectrophotometry at 486 nm by the rate of 2,6-dimethpxyphenol (Sigma, United States) oxidation [10]; activity of laccase, by the rate of oxidation of 2,2-azino-bis(3-ethylben-

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zothiazolin-6-sulfonate) (ABTS; Sigma) to a stable cation radical at 436 nm [11].

Isolation and purification of the enzymes. To isolate Mn peroxidase and laccase, 36-h bacterial culture was used. The cells were sedimented for 15 min at 7000 *g* on a K-24 centrifuge (MLV, Germany). The supernatant was used for further purification. The first stage included fractional precipitation of proteins from the culture liquid with 0–85% ammonium sulfate; the second stage consisted of selective sorption of admixtures on a CaF_2 gel (basing on the method suggested by Novakovskii and coauthors [12]). Gel permeation chromatography was then carried out on a Sephadex G75 column (Sigma-Aldrich, Sweden). Elution of protein fractions with 0.1 M NaCl was monitored using Uvicord S-II (LKB, Sweden) at λ = 280 nm. The next stage included ion-exchange chromatography on a Toyopearl DEAE 650M (Tosoh, Japan) adsorbent, particle size 10–40 μm. Bound proteins were eluted from the adsorbent in a step gradient from 0 to 1 M NaCl. Fractions with Mn peroxidase and laccase activities were dialyzed against water and used in the experiments. Protein composition at each stage of purification, as well as the homogeneity of the enzyme preparations and their molecular mass, were determined with SDS-PAGE according to Laemmli [13] in 12.5% polyacrylamide gel. At all stages of purification, laccase and Mn peroxidase activities were measured.

To study the role of phenol oxidases in the formation of gold nanoparticles, aqueous solutions of purified enzymes were incubated with 30 μM $HAuCl_4$ at room temperature for 24 and 48 h.

Transmission electron microscopy (TEM). Samples of whole bacterial cells and culture liquid were studied using the negative staining technique. The material was applied to nickel grids coated with formvar (from a 1% solution in dichloroethane). After drying, the cells were stained with 1% aqueous uranyl acetate [14]. Microphotographs were obtained on a Libra 120 electron microscope (Carl Zeiss, Germany) at 120 keV.

Characterization of nanoparticles. To analyze absorption spectra of the nanoparticles, Specord 250 (Analytik Jena, Germany, 190–1100 nm) was used. The size, shape, and relative abundance of electron-dense nanoaggregates were determined using TEM images obtained on a Libra 120 electron microscope (Carl Zeiss). To measure the zeta potential, average diameter, and size distribution of the Au^0 particles, the Zetasizer Nano ZS (Malvern, United Kingdom) was used.

RESULTS AND DISCUSSION

Effect of a gold-containing compound $HAuCl_4$ on bacterial growth. Initially, the sensitivity of the strains to chloroauric acid, effect of the compound on growth characteristics, and accumulation of bacterial biomass during growth in a liquid nutrient medium were tested. An aqueous solution of $HAuCl_4$ was added to a 24-h culture to a final concentration of 5–500 μM and, after 18 h incubation, the concentrations characterizing the level of toxicity of the gold-containing compound were determined. The minimal growth-inhibiting concentration was 40 μM, no growth was observed at 100–500 μM, and at 5 μM, the growth rate practically did not differ from that in the control.

It was noted that upon cultivation of *A. brasilense* Sp245 and Sp7 in a liquid synthetic medium in the presence of $HAuCl_4$ at concentrations of 5–30 μM, a pale violet coloration developed in the medium on the second day; with time (72 h), the coloration became more intense. According to the literature data, such a coloration is characteristic of bacteria, algae, and fungi growing on gold-containing media and is indicative of gold nanoparticle accumulation in the culture liquid [15]. Control bacterial cultures were of creamy white color during the whole cultivation process, with the medium not changing its color. In an uninoculated synthetic medium of the same composition supplemented with $HAuCl_4$ at concentrations of 5–500 μM, no change of color or sediment formation occurred during 72 h of incubation. This evidenced that in this case the process of gold nanoparticle formation was not associated with chemical reduction of gold by components of the cultivation medium.

For further experiments, we chose an $HAuCl_4$ concentration of 30 μM at which no growth inhibition occurred, meanwhile the cultivation medium changed its color to pale violet.

Transmission electron microscopy. Using TEM and negative staining technique, the culture liquid and bacterial cells of two *A. brasilense* strains, Sp245 and Sp7, grown for two (Figs. 1a, 1b) or three days in the presence of 30 μM $HAuCl_4$ were studied. As follows from the images, electron-dense formations of different shapes and largely varying size were observed in the culture liquid and near some bacterial cells. Increase in the incubation duration resulted in an increase in nanoparticle number.

To study the nanoparticles of gold reduced by *A. brasilense* Sp245 and Sp7, the medium was separated from bacterial cells by filtration (pore size of 0.45 μm) and lyophilized. Then, the nanoparticle-containing lyophilized material was resuspended in distilled water and applied onto coated nickel grids. Gold particles separated by filtration represented a rather large number of nanospheres and nanoprisms from 5 to 300 nm in size (Fig. 2). Diameter of the nanospheres varied between 5 and 50 nm, with particles of 30 nm in size predominant. Flat and three-dimensional triangles, pentagons, and tetrahedrons were of 5 to 300 nm in size.

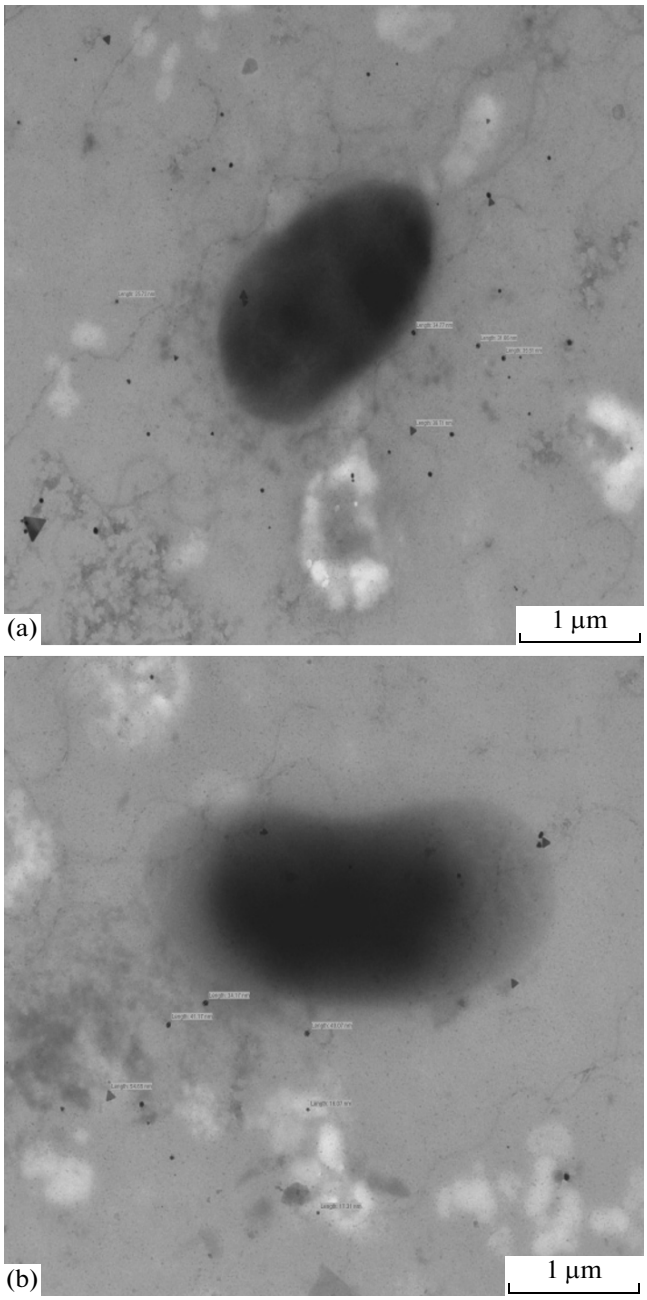

Fig. 1. Negative staining (TEM) of *A. brasilense* Sp245 (a) and Sp7 (b) after 48 h cultivation in a liquid synthetic medium in the presence of 30 μM $HAuCl_4$.

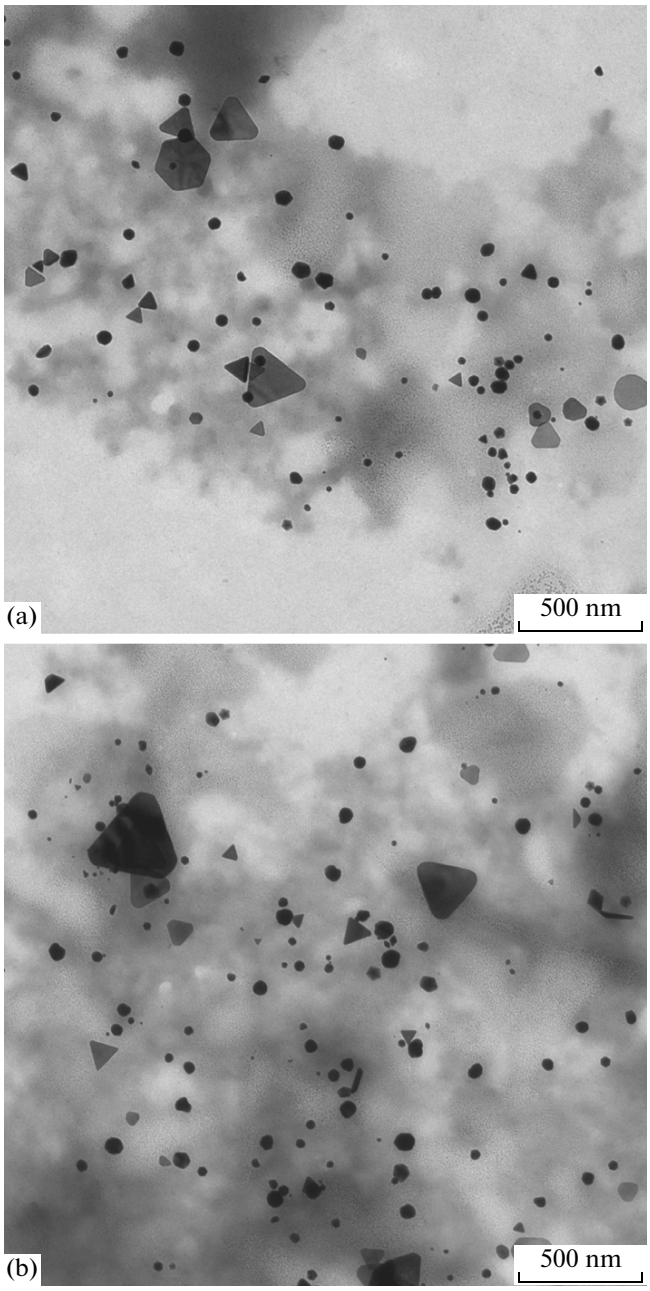

Fig. 2. Transmission electron microscopy of gold nanoparticles isolated from the culture liquid of *A. brasilense* Sp245 (a) and Sp7 (b) on the third day of cultivation in a liquid synthetic medium in the presence of 30 μM $HAuCl_4$.

Ultraviolet spectroscopy is one of the major methods to study the processes of formation and stability of metallic nanoparticles in aqueous solutions [16]. Figure 3 demonstrates absorption spectra of the nanoparticle suspensions, which reflect formation of nanoparticles at $HAuCl_4$ concentration of 30 μM in a bacterial culture incubated for two or three days. While the nanoparticles formed by both the second and the third day of incubation had the main absorption maximum in the red region of the spectrum at 550 nm, after 72 h the amount of the nanoparticles was much higher and the colloid composition was more heterogeneous.

Biological generation of gold nanoparticles with phenol-oxidizing enzymes of *A. brasilense*. Experimental works of the past decade evidenced the ability of some microorganisms to reduce gold ions employing

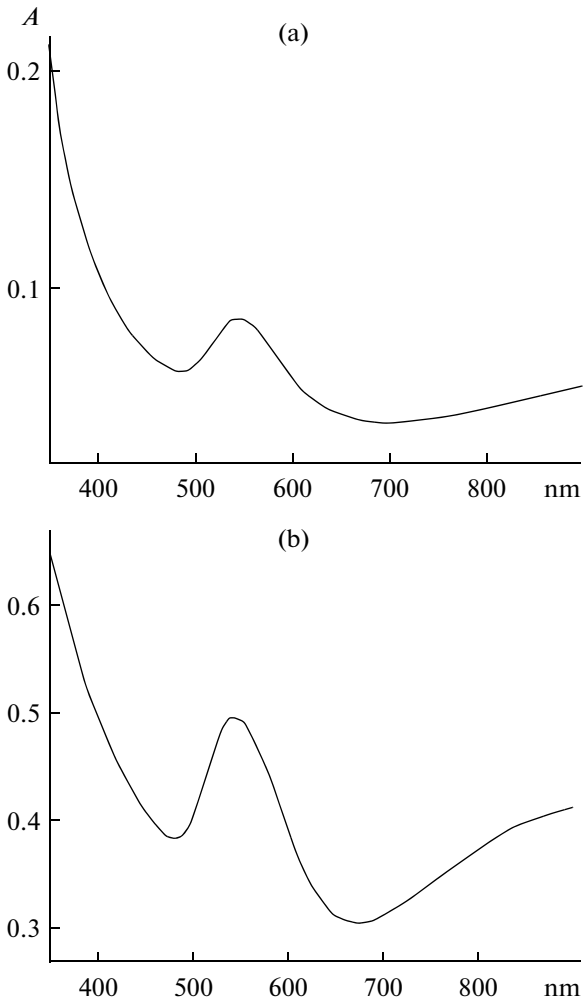

Fig. 3. Absorption spectra of gold nanoparticles formed in the culture liquid of *A. brasilense* Sp245 on the second (a) and third (b) days of cultivation in a liquid synthetic medium in the presence of 30 μM $HAuCl_4$.

the proteins they synthesize. For example, metal-reducing bacteria of the genus *Shewanella* use their cell wall proteins to produce gold nanoparticles [6]. It is supposed that reduction of gold ions to the elementary form in a number of bacteria is performed by NADH-dependent enzymes [17], for example, by NADH-dependent nitrate reductase, as in *Rhodopseudomonas capsulata* [4]. The hypothesis that oxidoreductases of lower fungi [18] and laccases and ligninases of the basidiomycete *Planerochaete chrysosporium* [8] are involved in the mechanism of biological reduction of gold-containing compounds is of interest. Relatively recently, we showed the presence of phenol-oxidizing activity in azospirilla [19]. We assumed that reduction of gold ions by azospirilla to the elementary state may proceed with the aid of the same phenol-oxidizing enzymes.

To confirm the hypothesis, extracellular laccases and Mn peroxidases were isolated from the culture liquid of azospirilla. Extracellular Mn peroxidases of *A. brasilense* Sp245 and Sp7 are single-subunit proteins of approximately the same molecular mass in the range of 42–44 kDa, and laccases, of 60–62 kDa. Specific activity of the isolated Mn peroxidases and laccases was 106.4 and 3.1 U/mg protein for *A. brasilense* Sp245 and 93.7 and 9.2 U/mg protein, for *A. brasilense* Sp7, respectively. Aqueous solutions of purified enzymes were incubated with 30 μM $HAuCl_4$ at room temperature. After 24 h, the mixture was colored, indicating the formation and accumulation of gold nanoparticles. The suspension containing a preparation of laccase had a blue coloration and that containing Mn peroxidase, had a violet coloration. After 48-h incubation, the color intensity increased. The formation of gold nanoparticles by *Rhodopseudomonas capsulata* occurred within the same time interval [4]. It should be noted that the proteins of azospirilla possessing no phenol oxidase activity—for example, lectins—which we had isolated from the azospirillar cell surface [20], as well as bovine serum albumin used as the control, did not reduce gold to the elementary state.

As follows from Fig. 4, the nanoparticles formed with bacterial Mn peroxidase within 24 h were mainly nanospheres from 5 to 15 nm in diameter and, as a minor component, nanoprisms. Meanwhile, the nanoparticles formed after 48 h incubation were electron-dense formations of spherical, triangular, or tetrahedral shape from 5 to 300 nm in size. No difference between strains was observed in the effect of the enzymes. Extracellular formation of heterogeneous suspensions of gold nanoparticles with predomination of nanotriangles was noted also for *Escherichia coli* [21]. The particles formed with the preparations of laccase from *A. braslense* Sp245 and Sp7 were conglomerates of irregular shape (Fig. 4c). The formation of similar gold nanoparticles was observed in *Rhodopseudomonas capsulata* [22].

The size of the reduced gold nanoparticles was also determined by dynamic light scattering (Figs. 5, 6). The zeta potential of colloidal gold reduced upon incubation for 48 h was −5.08 and −3.34 mV for Mn peroxidase and laccase, respectively.

Proposed mechanism of bioreduction of gold by phenol oxidases of *A. brasilense*. Despite the observed ability of a number of bacteria to reduce gold to the elementary state, it remains unclear how exactly the processes of bioreduction of gold-containing compounds proceed in bacterial cells. Our understanding of the importance of such properties in bacteria is also rather obscure. Similar to the soil bacteria *A. brasilense,* many microorganisms are known to preserve their viability at high concentrations of metal ions in the environment [23]. Most probably, the abil-

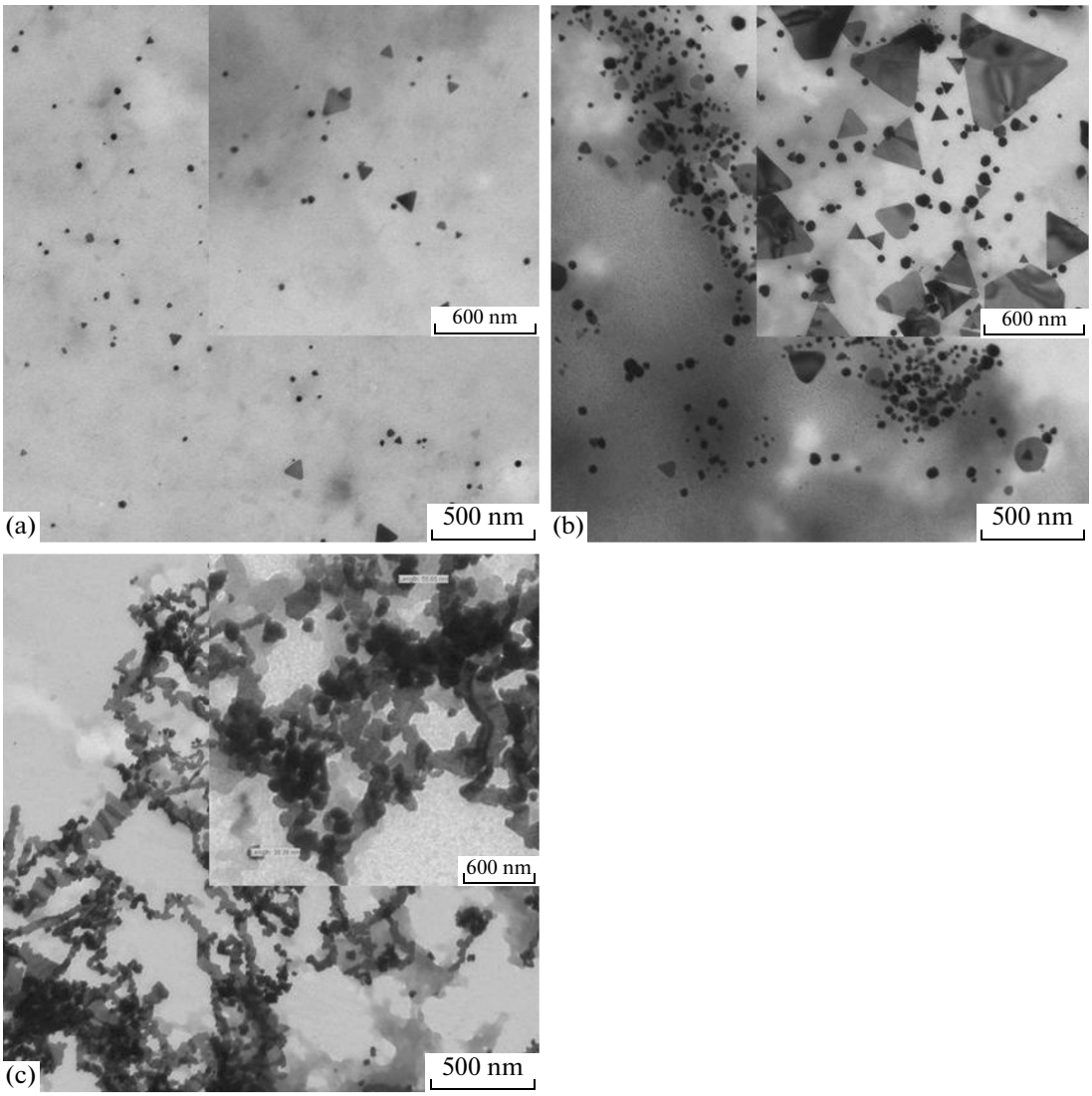

Fig. 4. Transmission electron microscopy of gold nanoparticles isolated from a 30-μM solution of $HAuCl_4$ with *A. brasilense* Sp245 Mn peroxidase after 24 h (a) and 48 h (b) incubation and with laccase, upon 48 h incubation (c).

ity of azospirilla to form gold nanoparticles from chloroauric acid is also a protective mechanism. Apparently, bioreduction with the formation of Au^0 is necessary to decrease the toxic effect of high concentrations of the substance. In different microorganisms, different biomolecules are probably involved in the process. Moreover, one may not exclude that the mechanisms of extracellular and intracellular particle formation may be different [24].

It is assumed that the extracellular mechanisms of nanoparticle formation are mainly associated with the activity of nitrate reductases, although these assumptions are of hypothetical nature [4, 7].

On the basis of our results demonstrating the capability of *Azospirillum* phenol oxidases for reduction of Au^{3+} to Au^0, we propose the probable schemes of reactions. Mn peroxidase contains a protoporphyrin IX with a Fe^{3+} ion [25] as a prosthetic group and, similar to other peroxidases, may be reduced to the ferriform, with Fe^{2+} oxidation state, at low pH values [26]. We assume that extracellular reduction of $AuCl_4^-$ performed by the prosthetic group of the enzyme is a bypass of the main catalytic cycle. This possibility is supported by the results of the experiment in which Mn^{2+} ions and hydrogen peroxide, the main substrates of the enzyme, were not introduced in the reaction mixture. As a result of the redox reaction, the formation of elementary gold occurred and the enzyme was transferred to a so-called quiescent fully oxidized form. The subsequent formation of morphologically different nanoparticles in a mixture containing Mn peroxidase follows the scheme (Fig. 7).

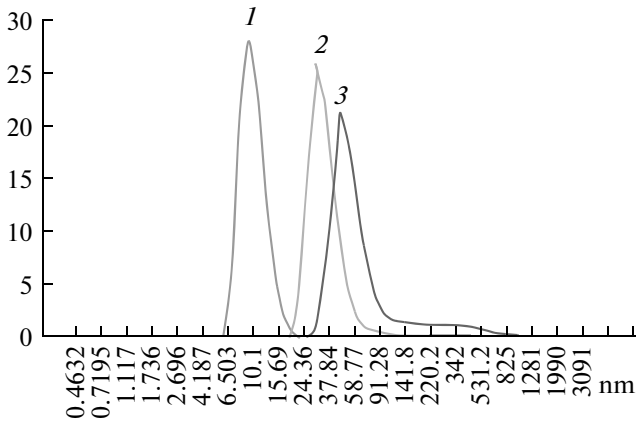

Fig. 5. Average diameter of Au^0 particles formed by phenol-oxidizing enzymes of *A. brasilense* from $HAuCl_4$: laccase, 48 h incubation (*1*) and Mn peroxidase, 24 h (*2*) and 48 h incubation (*3*).

The reduction of gold with laccase presumably occurs indirectly, via formation of exogenous peroxide by the enzyme. When laccase interacts with molecular oxygen in the absence of reduced substrates, hydrogen peroxide is formed in one of the four active centers of the enzyme [27]; this hydrogen peroxide is involved in reduction of gold. The proposed hypothesis on the pathway of gold nanoparticle formation stemmed from comparison of our results on incubation of $HAuCl_4$ with our preparation of bacterial laccase and the results of the work of Pestovskii [28], who noted formation of conglomerates of nanospheres, similar to those we observed (Fig. 4c) in the course of reduction of gold by hydrogen peroxide.

Thus, in the present work, data on the formation of gold nanoparticles in *A. brasilense* cultures were obtained. It was found that the formation of colloidal gold proceeds extracellularly, similar to *Lactobacillus* sp., *Rhodopseudomonas capsulata,* and *Pseudomonas aeruginosa* [3–5]. For the first time, the role of phenol oxidizing enzymes—that is, Mn peroxidase and laccase—in the process of gold reduction was demonstrated. Schemes of the mechanisms of gold reduction by phenol oxidases of azospirilla are proposed.

Application of plant and microbial cell cultures to the production of colloidal gold becomes a new direction in nanobiotechnology [1]. The availability, simplicity, and safety of biological production of gold

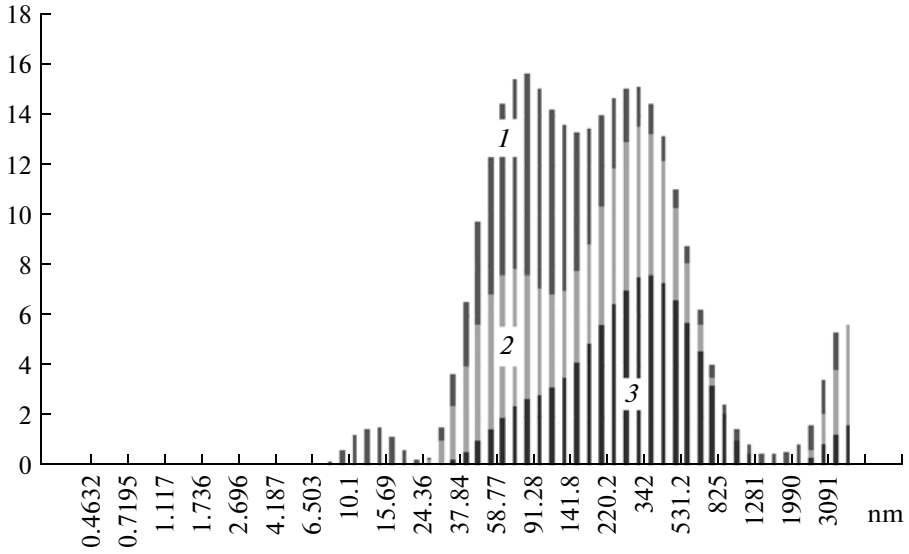

Fig. 6. Size distribution of Au^0 particles formed by phenol-oxidizing enzymes of *A. brasilense* from $HAuCl_4$: laccase, 48 h incubation (*1*) and Mn peroxidase, 24 h (*2*) and 48 h incubation (*3*).

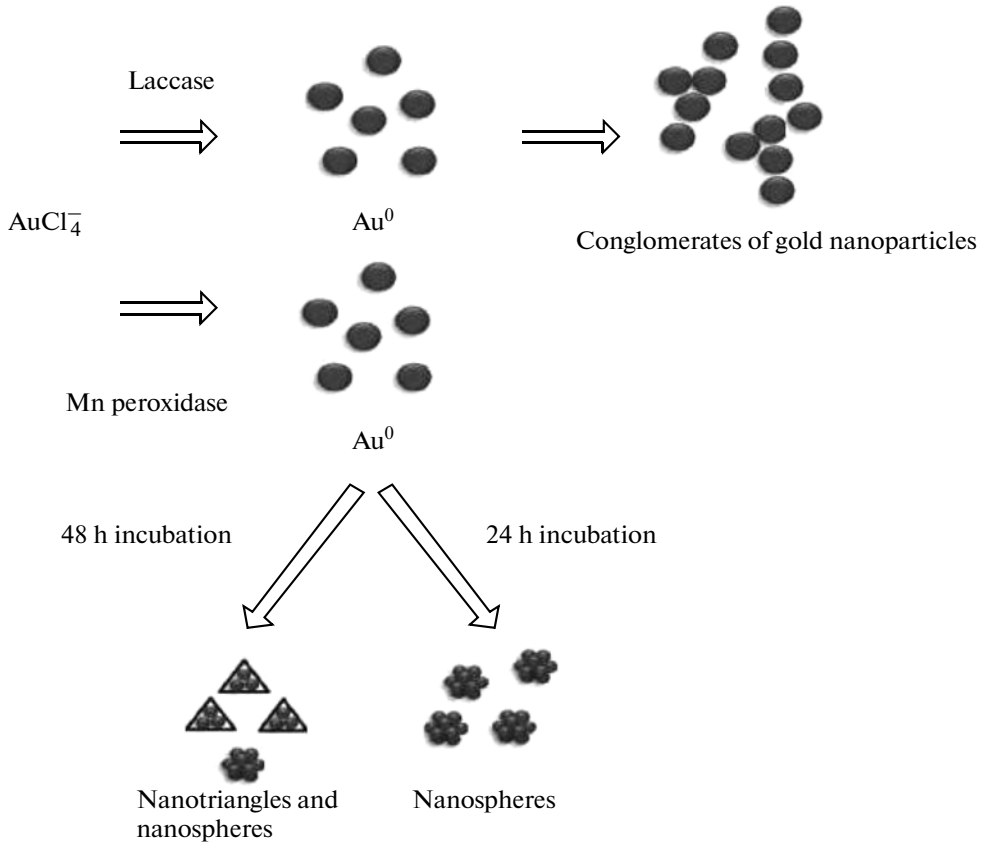

Fig. 7. Hypothetical mechanism of gold nanoparticles formation under the effect of phenol oxidases of azospirilla.

nanoparticles by the associative soil bacterium *A. brasilense* make this method promising for environmentally friendly production of nanoparticles.

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