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Gold(III) Reduction by the Rhizobacterium *Azospirillum brasilense* with the Formation of Gold Nanoparticles

Anna V. Tugarova · Andrei M. Burov · Marina M. Burashnikova · Alexander A. Kamnev

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Abstract For the soil nitrogen-fixing bacterium *Azospirillum brasilense*, the ability to reduce $[AuCl_4]^-$ and to form gold nanoparticles (GNPs) has been demonstrated, with the appearance of a mauve tint of the culture. To validate the shapes and chemical nature of nanoparticles, transmission electron microscopy (TEM) and X-ray fluorescence analysis were used. For the widely studied agriculturally important wild-type strains *A. brasilense* Sp7 and Sp245, GNPs formed after 10 days of incubation of cell biomass with 0.25 mM $[AuCl_4]^-$ were shown (using TEM) to be mainly of spherical form (5 to 20 nm in diameter), with rare occasional triangles. In the course of cultivation with $[AuCl_4]^-$, after 5 days, a mauve tint was already visible for cells of strain Sp245.5, after 6 days for Sp245 and after 10 days for Sp7. Thus, for the mutant strain Sp245.5 (which has significant differences in the structure and composition of cell-surface polysaccharides as compared with Sp245), a more rapid formation of GNPs was observed. Moreover, their TEM images (also obtained after 10 days) showed different shapes: nano-sized spheres, triangles, hexagons and rods, as well as larger round-shaped flower-like nanoparticles about 100 nm in size. Since by the time of GNP formation in our experiments the cells were found to be already not viable, this confirms the dominating role of cell surface structure and chemical composition in shaping the GNPs formed in the course of $[AuCl_4]^-$ reduction to Au^0. This finding may be useful for understanding the natural biogeochemical mechanisms of gold reduction and formation of GNPs involving microorganisms. The data obtained may also help in developing protocols for environmentally friendly synthesis of nanoparticles and possible use of bacterial cells with modified surface structure and composition for their fabrication.

A. V. Tugarova (✉) · A. M. Burov · A. A. Kamnev (✉)
Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, 410049 Saratov, Russia
e-mail: tugarova_anna@mail.ru
e-mail: aakamnev@ibppm.sgu.ru

A. A. Kamnev
e-mail: a.a.kamnev@mail.ru

M. M. Burashnikova
Institute of Chemistry, N.G. Chernyshevsky Saratov State University, 410012 Saratov, Russia

Introduction

The participation of microorganisms in transformations of gold compounds with various Au oxidation states in the environment (i.e. in the biogeochemical cycle of gold) has been discussed in numerous reports including reviews [13, 35]. Microorganisms are considered to play a role in processes leading to the formation of gold ores and gold nanoparticles occurring in nature [16]. The biotransformation of gold includes its reduction from Au^{III} compounds to metallic gold (Au^0). The formation of gold nanoparticles (GNPs) has been reported for a range of microorganisms including bacteria and fungi [6, 9, 34]. The GNP synthesis can occur both extracellularly [18, 31] and intracellularly [1, 26].

The potential of microorganisms to reduce gold(III) and to form GNPs is of interest also for the developing trend of 'green chemistry' [6]. Its essence is the use of various living organisms, in particular, many species of bacteria [15, 33], for the 'green synthesis' of nanoparticles instead of physicochemical methods, which can often be costly and require hazardous reagents.

The bacteria of the genus *Azospirillum*, and particularly the *A. brasilense* species, belong to most widely studied rhizobacteria and are capable of forming associative symbioses with many higher plants. They can promote plant growth and development via the production of

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phytohormones (particularly auxins), atmospheric nitrogen fixation and other mechanisms [3].

For the two strains, *A. brasilense* Sp7 and Sp245, that differ by the ecological niches which they can occupy in the rhizosphere and by their responses to stresses [3, 4, 20–22], the ability to reduce selenite (SeO_3^{2-}) to elementary selenium has recently been reported [37]. Using transmission electron microscopy (TEM) and X-ray fluorescence analysis (XFA), both strains were shown to accumulate elementary red selenium in the form of nanoparticles 50 to 400 nm in diameter. In this work, we studied the ability of *A. brasilense* to reduce gold(III) and to form GNPs using TEM and XFA. In order to test the influence of bacterial cell surface on the processes of GNP formation and on their shape, a mutant strain was used which has an altered surface composition and structure of cell-surface polysaccharides [23] as compared with wild-type strain Sp245.

Materials and Methods

Strains and Cultivation Conditions

A. brasilense wild-type strains Sp7 ([36]; ATCC 29145 [14]) and Sp245 [2], taken from the Collection of the IBPPM RAS, Saratov, were used in experimental work, together with a mutant strain of the latter, Sp245.5, kindly provided by our colleagues from the Laboratory of Microbial Genetics of the IBPPM RAS [23].

Cultures were grown on liquid or agar-containing (2 %) synthetic malate medium [7] modified to the following composition: 3.0 g l^{-1} K_2HPO_4, 2.0 g l^{-1} KH_2PO_4, 0.1 g l^{-1} NaCl, 3.76 g l^{-1} malic acid, 2.24 g l^{-1} NaOH, 0.5 g l^{-1} NH_4Cl, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.02 g l^{-1} $CaCl_2$, 0.02 g l^{-1} $FeSO_4 \cdot 7H_2O$ and 0.002 g l^{-1} $Na_2MoO_4 \cdot 2H_2O$ (pH 6.8–7.0). Liquid cultures were grown for 18–20 h with aeration on a shaker (200 rpm); the cultures on the semisolid medium (on Petri dishes) were grown for 3 days. Bacterial cultivation was performed at 31 °C.

Determination of the Ability to Reduce Gold(III)

To check for the ability of the cultures to reduce gold(III), the following scheme was used (all operations were carried out under sterile conditions). The cultures grown overnight were collected by centrifugation (10 min; 10,000×*g*). The collected cells were washed twice with physiological solution (0.85 % NaCl) in order to exclude any possible influence of substances excreted into the growth medium by growing bacteria, as well as of culture components, on the processes of gold(III) sorption and reduction. The washed samples were resuspended and brought to the same optical density (1.0) at 600 nm. Dissolved $H[AuCl_4]$ (0.50 mM; prepared from $H[AuCl_4] \cdot 3H_2O$, Sigma–Aldrich) was added at the 1:1 ratio (final Au concentration 0.25 mM; pH 3.8). The cells in the solution were thermostatted at 31 °C and incubated under those conditions for 10 days.

Transmission Electron Microscopy

The samples prepared as above (after 10 days of incubation with $H[AuCl_4]$) were carefully resuspended prior to being applied to formvar-coated grids. Microphotographs were obtained using a Libra 120 electron microscope (Carl Zeiss, Germany) at 120 kV.

X-ray Fluorescence Analysis

Elemental X-ray fluorescence analysis (XFA) of the dried biomass samples was carried out using an EDX-720 energy dispersive X-ray fluorescence spectrometer (Shimadzu, Japan). Measurements were carried out using the cuvette mode, with the range of detected elements from Na to U and with air as the medium. The content of the elements was determined using the method of fundamental parameters using the software package bundled with the spectrometer. Gold was detected by its lines at 9.714 ($L_{\alpha 1}$, main line), 11.443 ($L_{\beta 1}$), and 13.383 keV ($L_{\gamma 1}$).

Results and Discussion

In a few days of incubation with $[AuCl_4]^-$, the cultures of all the strains showed mauve tints: after 5 days, a mauve tint was visible for cells of strain Sp245.5, after 6 days for Sp245, and after 10 days for Sp7. After 10 days, the tint was visible also in the supernatant of strain Sp245.5. The staining may be attributed to the formation of GNPs. In the course of incubation of microorganisms in the presence of $[AuCl_4]^-$, the appearance of various tints from wine red to mauve and purple is documented to be a sign of gold(III) reduction with the formation of various nanoparticles, which has been reported for bacteria, algae and fungi [1, 5].

On the tenth day, TEM studies were performed. For strains Sp245 and Sp7, TEM showed that the cells contained electron-dense spheroids 5 to 20 nm in size (Fig. 1a–d) which were absent in the control (grown without $[AuCl_4]^-$, not shown). Other shapes of nanoparticles, mainly triangles, were rare. Nanoparticles were observed both in the vicinity of cell walls and extracellularly.

For the mutant strain, Sp245.5, more heterogeneous GNPs were observed. Along with spheroid-shaped GNPs 5 to 20 nm in size, there occurred nano-sized rods, triangles and hexagonal particles, as well as relatively large round particles up to 100 nm in size (Fig. 1e,f). As mentioned

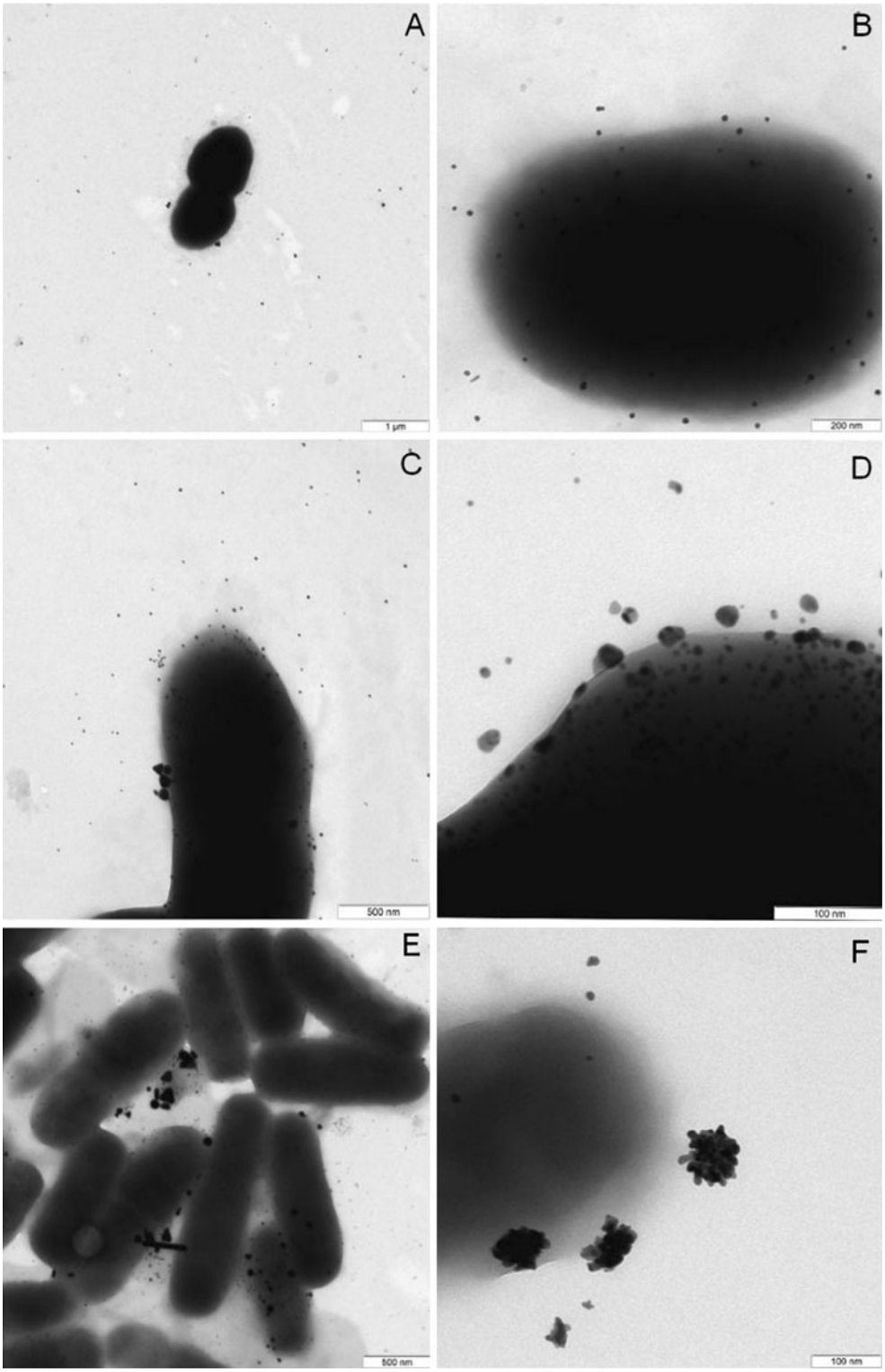

Fig. 1 Transmission electron microscopy of cells of *A. brasilense* Sp7 (**a**, **b**), Sp245 (**c**, **d**) and Sp245.5 (**e**, **f**) after incubation of live cells for 10 days with 0.25 mM $H[AuCl_4]$. Scale bars: **a** 1 μm, **b** 200 nm, **c**, **e** 500 nm, **d**, **f** 100 nm

above, strain Sp245.5 showed a higher gold(III) reduction rate (a mauve tint appeared on the fifth day) and gave unusual aggregates. The nanoparticles were very similar to flower-like Au nanoparticles ('nanoflowers') with rough surfaces (such as those reported in [39]). Similar to other particles, they were localised both inside and outside the cells. In the literature, we have found a single report on the synthesis of similar flower-like GNPs by extracts of the mushroom *Volvariella volvacea* [34], while there were no reports on flower-like GNPs synthesised by bacteria.

It has to be mentioned that strain Sp245.5 is a mutant of Sp245 (RP4) lacking RP4, p85 and p120 with the formation of a new ca. 300-MDa replicon after long-term storage in a rich medium, Tc^S, Km^S [23]. The mutant strain has an altered composition of cell-surface polysaccharides which can primarily interact with metal ions [17]. For strain Sp245 and its mutant Sp245.5, significant differences in the structures of the O-specific polysaccharides of their lipopolysaccharides were found [11, 12]. The repeating fragment of the O-specific polysaccharide in *A. brasilense* Sp245 is a pentasaccharide consisting of *D*-rhamnose residues [11]. For strain Sp7, its O-specific polysaccharide also consists of *D*-rhamnose as well as of other sugars: fucose, galactose, etc. [38]. The O-specific polysaccharides of both wild-type strains are neutral by charge. Both strains also produce polysaccharides which bind the fluorochrome dye Calcofluor (Cal^+ phenotype) [8]. In Sp245.5, the repeating fragment of the O-specific polysaccharide is represented by a disaccharide formed by the residues of *N*-acetyl-*D*-galactosamine and *N*-acetyl-*D*-mannosamine uronic acid (the latter is a sugar unusual for azospirilla) [12]. Thus, *A. brasilense* strain Sp245, similar to Sp7 [8], produces polysaccharides which bind Calcofluor (Cal^+ phenotype) [24], whereas its mutant Sp245.5 has the Cal^- phenotype [23].

In the literature, there are several reports on the synthesis of GNPs using polysaccharides. A method involving GNP synthesis on natural cellulose fibres was reported in [10]. The in situ synthesis method included electrostatic adsorption of negatively charged metal complex ions onto the cationically modified cellulose surfaces. Chemical reduction of the metal ions with $NaBH_4$ led to the formation of metal nanoparticles on the substrates. The first step of this process occurs also in the case of bacterial gold(III) reduction, i.e. binding of negatively charged gold complex ions to positively charged functional groups of the bacterial cell surface.

The formation of GNPs was also described using gold(III) reduction with aminodextran [32]. The GNPs shape depended on the pH; at low pH (~4), the reduction gave Au crystals of various shapes, whereas at pH~10, gold nanospheres of a similar diameter were formed. In that case, the modified polysaccharide was directly involved in gold(III) reduction, leading to the formation of GNPs.

For *Pseudomonas aeruginosa*, the role of the chemical environment and physiological state in metal immobilisation was investigated [17]. Modification of the lipopolysaccharide as a function of changing conditions was shown to influence binding of metal ions, in particular, of gold. These

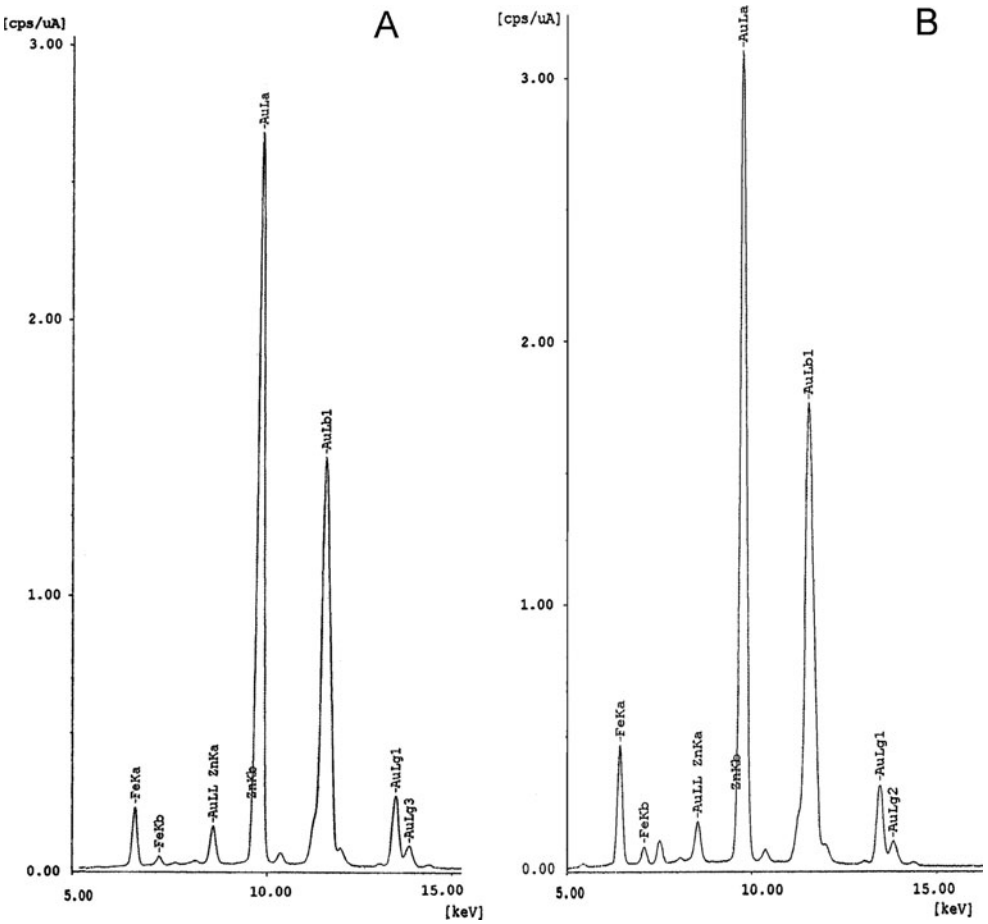

Fig. 2 X-ray fluorescence analysis of dry biomass samples of *A. brasilense* strains Sp245 (**a**) and Sp245.5 (**b**) after incubation of live cells for 10 days with 0.25 mM $H[AuCl_4]$. Gold is featured by its main lines at 9.714 ($L_{\alpha 1}$), 11.443 ($L_{\beta 1}$) and 13.383 keV ($L_{\gamma 1}$)

results may account for the accumulation of metals at the bacterial cell surfaces and for differences in metal accumulation for different microbial consortia. Cell surfaces and their extracellular biopolymers are capable of binding significant quantities of metal ions owing to a high concentration of reactive ligands, in particular, carboxyl and phosphoryl groups [17].

The lipopolysaccharide chemotype influences both adsorption and precipitation of metal ions. According to the literature, the reduction process can pass the following steps. Gold(III) complex ions, $[AuCl_4]^-$, adsorb at the surface of bacterial cells in acidic media with the involvement of positively charged protonated oxygenous and nitrogenous active functional groups contained in cell surface biomacromolecules [30]. On the further step, reducing sugars mediate the rapid reduction of Au^{III} to Au^{I}, followed by a slow reduction to Au^0. In the course of Au^{III} bioreduction, free aldehyde groups of the cyclic hemiacetalic hydroxyl from various reducing sugars can be oxidised to carboxyl with the bound Au^{III}, which is finally reduced in situ to Au^0 [30]. The aforementioned data correlate well with the differences in GNP synthesis between strains Sp7 and Sp245, on the one hand, and the mutant strain Sp245.5 significantly differing in the polysaccharide composition from the latter, on the other hand.

As was mentioned above, gold(III) reduction to Au^0 with the synthesis of GNPs was reported for various microorganisms: cyanobacteria [27], *Aspergillus oryzae* [5], *P. aeruginosa* [18], the thermophilic bacterium *Geobacillus stearothermophilus* [31], the actinomycete *Rhodococcus* species [1] and the anaerobic bacterium *Shewanella algae* [26]. Similar results were obtained also for silver [25]. Microorganisms were shown to facilitate the formation of secondary gold in placer deposits and of gold nuggets [27, 28].

Since the main habitat of azospirilla is the rhizosphere of higher plants and soil, their participation in the biogeochemical cycle of gold is also quite possible. In addition, the attention of researchers has recently been drawn to bacteria as a means of fabrication of GNPs [6, 25].

In order to validate the nature of the nanoparticles formed in the bacterial cultures (see Fig. 1), X-ray fluorescence analysis of dry biomass samples was performed for strains Sp245 and Sp245.5 which had shown different shapes of particles. The cells incubated with $H[AuCl_4]$ for 10 days showed significant Au accumulation (Fig. 2). The presence of gold was determined by its lines at 9.714 ($L_{\alpha 1}$, main line), 11.443 ($L_{\beta 1}$), 13.383 keV ($L_{\gamma 1}$) and some others. Note that along with most intensive Au lines, a few additional relatively weak lines are observed in Fig. 2 for Fe and Zn, which are common microelements found in bacterial cells and, in particular, in *A. brasilense* [19, 21].

It has to be noted that in our TEM images (see Fig. 1), an increased electron density of the whole cells was observed (the samples had been prepared without using uranyl acetate as a contrasting agent). Such enhancement in this case may imply localisation and accumulation of gold ions and elementary gold inside the cells of azospirilla, suggesting their role as natural matrices for reducing gold ions.

Our attempt to cultivate the bacteria on the semisolid medium after 10 days in the presence of 0.25 mM $[AuCl_4]^-$ showed the absence of viable cells. Cell viability was affected by two parameters, the $[AuCl_4]^-$ concentration and pH. Thus, the processes of biosorption and reduction of $[AuCl_4]^-$ by azospirilla are evidently related to bacterial cell components, but not to the metabolism of live cells. As is known, gold(III) compounds are relatively toxic to bacteria [28]. It has also been shown that the processes of metal ion biosorption, reduction and formation of nanoparticles often occur on dead cells [28–30].

Note that the participation of some enzymes of azospirilla, either extracellular or intracellular, in gold(III) reduction cannot be excluded. Nevertheless, in this work, we have shown that differences in cell surface structures, namely in polysaccharide components, influence the formation of differently shaped GNPs (nano-sized spheres, triangles, hexagons, rods, and flower-like nanoparticles). This finding may be useful for understanding natural mechanisms of gold reduction and formation of GNPs involving microorganisms. On the other hand, the data obtained may help in developing protocols for environmentally friendly synthesis of nanoparticles and possible use of bacterial cells for their fabrication.

Thus, we have demonstrated the ability of *A. brasilense* to reduce $H[AuCl_4]$ with the formation of GNPs, which has not yet been reported for bacteria of the genus *Azospirillum*. As their natural habitat is the rhizosphere and soil, azospirilla can contribute to gold ion reduction under natural conditions and participate in the processes of geological rock formation. The results on the formation of GNPs of different shapes may also be of interest for nanobiotechnology.

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