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EXPERIMENTAL ARTICLES

The Production of Phenazine Antibiotics by the *Pseudomonas aureofaciens* Strain with Plasmid-Controlled Resistance to Cobalt and Nickel

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Abstract—Plasmid pBS501, responsible for the resistance of the wild-type *Pseudomonas* sp. BS501(pBS501) to cobalt and nickel ions, was conjugatively transferred to the rhizosphere *Pseudomonas aureofaciens* strain BS1393, which is able to synthesize phenazine antibiotics and to suppress a wide range of phytopathogenic microorganisms. The transconjugant *P. aureofaciens* BS1393(pBS501) turned out to be resistant to cobalt and nickel with an MIC of 8 mM. When grown in a synthetic medium with 0.25 mM cobalt, the transconjugant accumulated 6 times more cobalt than the wild-type strain BS501(pBS501) (1.2 versus 0.2 μg Co/mg protein). Electron microscopic studies showed that cobalt accumulates on the surface of transconjugant cells in the form of electron-opaque granules. In a culture medium with 2 mM cobalt or nickel, strain BS1393 produced phenazine-1-carboxylic acid in trace amounts. The transconjugant *P. aureofaciens* BS1393(pBS501) produced this antibiotic in still smaller amounts. Unlike the parent strain BS1393, the transconjugant *P. aureofaciens* BS1393(pBS501) was able to suppress in vitro the growth of the phytopathogenic fungus *Gaeumannomyces graminis* var. *tritici* 1818 in a medium containing 0.5 mM cobalt or nickel.

Key words: phenazines, rhizosphere *Pseudomonas*, plasmid, heavy metals.

Heavy metals often contaminate agricultural areas treated with pesticides and fertilized with sewage sludge. The contamination of soil by heavy metals adversely affects soil and rhizosphere microbiotas, a fact that should be taken into account when employing plant growth–promoting rhizobacteria (PGPR) in agriculture.

It is known that metals are necessary for the normal metabolism of microorganisms as microelements, as well as for the production of secondary metabolites. The addition of trivalent iron to the cultivation medium of the PGPR *Pseudomonas chlororaphis* PCL1391 was found to enhance the synthesis of phenazine-1-carboxamide [1]. Zinc ions stimulated the production of phenazine-1-carboxylic acid (P1CA) by the *P. fluorescens* strain 2-79 [2]. Cobalt at a concentration of 0.1 mM and iron ions at a concentration of 1 mM stimulated the synthesis of pyoluteorin in the *P. fluorescens* strain CHA0 [3]. However, little is known about the effect of high concentrations of heavy metals on the growth of rhizosphere pseudomonads and the synthesis of metabolites involved in their interactions with host plants and phytopathogenic microorganisms.

The aim of this work was to obtain a cobalt- and nickel-resistant strain of the PGPR *P. aureofaciens* BS1393 and to study its ability to produce phenazine antibiotics and to suppress the growth of phytopathogenic fungi in the presence of these heavy metals.

MATERIALS AND METHODS

Bacterial strains. The donor strain *Pseudomonas* sp. BS501(pBS501) contains the 65-kbp plasmid pBS501, which is responsible for bacterial resistance to cobalt and nickel [4]. The recipient strain *P. aureofaciens* BS1393 is the principle of the commercial preparation Pseudobacterin-2, which controls a wide range of phytopathogenic microorganisms. The antiphytopathogenic activity of *P. aureofaciens* BS1393 cells is due to the synthesis of the phenazine antibiotics P1CA, 2-hydroxyphenazine-1-carboxylic acid (2HP1CA), and 2-hydroxyphenazine (2HP) [5].

Growth media and cultivation conditions. The strains were grown on LB medium [6] and King B medium [7]. Cells for study by electron microscopy and atomic absorption spectroscopy were grown in a Tris-containing mineral medium (TM medium) [8] at 28–30°C. *P. aureofaciens* BS1393 and *Pseudomonas* sp. BS501(pBS501) were grown in the presence of 0.2% glucose or 0.2% sodium glutamate, respectively.

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Table 1. The minimal inhibitory concentrations of heavy metals for the donor, recipient, and transconjugant *Pseudomonas* strains grown in LB and TM media (data for the latter medium are given in parentheses)

Strain	Heavy metal, mM			
	cobalt	nickel	zinc	cadmium
Pseudomonas sp. BS501(pBS501)	8.0 (2.5)	8.0 (2.5)	4.0 (1.5)	0.5 (0.1)
P. aureofaciens BS1393(pBS501)	8.0 (2.5)	8.0 (2.5)	4.0 (2.5)	0.7 (0.5)
P. aureofaciens BS1393	2.0 (0.5)	2.0 (1.0)	4.0 (2.5)	0.7 (0.5)

Fungi were grown on dextrose–potato agar (DPA) [9] at 20–22°C. To assay antagonistic activity, the strains were grown in Kanner medium [9]. The minimal inhibitory concentrations of cobalt, nickel, cadmium, and zinc ions were determined by the serial dilution method. The heavy metals were added to LB and TM media at concentrations ranging from 0.1 to 10 mM, using 0.3 M stock solutions of $CoCl_2$, $NiCl_2$, $Cd(NO_3)_2$, and $ZnSO_4$ salts.

The plasmid pBS501 was conjugatively transferred on LB agar. The transfer lasted 18 h. Transconjugants were selected using TM medium with glucose and 2 mM $CoCl_2$.

Analysis of phenazines. The recipient strain BS1393 and its transconjugant BS1393(pBS501) were grown in the liquid King B medium with different concentrations of the heavy metals at 30°C for 2 days with aeration and then at 18°C for the next 2 days without aeration. Phenazines were extracted from the medium and analyzed by thin-layer chromatography (TLC) as described by Fernandez and Pizarro [10], using 60 Å TLC plates (Sigma-Aldrich).

Phenazines were also separated by reversed-phase high-performance liquid chromatography on a (3.9 × 50 mm) NOVA-PACK C_{18} column (Waters, United States), using a methanol–50 mM orthophosphoric acid (44 : 56) mixture as the mobile phase.

Tests for antagonistic activity. The indicator phytopathogenic fungus *Gaeumannomyces graminis* var. *tritici* (Ggt), strain 1818, was a gift from L. Thomashow, Washington State University, Pullman, Washington, United States. Cultures of strains BS1393 and BS1393(pBS501) 18 h old were plated onto Kanner agar containing 0.5 mM cobalt or nickel ions and incubated at 24°C for 24 h. Pieces of the Ggt 1818 mycelium were placed at the centers of agar plates with the aid of a drill for stoppers. The plates were incubated at 24°C for 5 days, after which the antagonistic activity of the bacterial strains was determined from the radius of the growth-inhibition zone of the indicator strain Ggt 1818.

Atomic absorption spectroscopy. Cells were grown in TM medium containing 0.25, 0.5, 1, 1.5, and 2 mM $CoCl_2$ for 18 h, washed twice with TM medium without cobalt, and precipitated by centrifugation. Alternatively, cells were grown in TM medium for 18 h, washed twice with fresh TM medium, incubated in TM medium with 5 mM $CoCl_2$ at 0°C for 2 h, and precipitated by centrifugation. The cell biomass was incinerated in 57% perchloric acid at 180°C, and the cobalt content of the cell ashes was determined using an AAS-5100/Zeeman atomic absorption spectroscope (Perkin-Elmer) operated on an acetylene–air mixture. The cobalt content was calculated per mg protein. The protein concentration was determined by the method of Lowry *et al.* [11].

Electron microscopy. Bacterial cells grown in TM medium with or without 2 mM $CoCl_2$ for 18 h were precipitated by centrifugation and washed twice with fresh TM medium. Thin sections were prepared by the Reynolds method [12] and analyzed with a JEM-100B electron microscope operated at a voltage of 60 kV.

RESULTS

Derivation of the Strain, P. aureofaciens BS1393(pBS501) Resistant to Heavy Metals

The rhizosphere strain *P. aureofaciens* BS1393 is sensitive to the heavy metal cations Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} (Table 1). To enhance its heavy-metal resistance, this strain was transformed by the conjugal transfer of plasmid pBS501 from the *Pseudomonas* sp. strain BS501(pBS501), resistant to cobalt and nickel with an MIC of 8 mM [4]. The inability of the donor strain to utilize glucose made it possible to select transconjugants using TM medium with glucose and 2 mM $CoCl_2$. The conjugal transfer rate of plasmid pBS501 into *P. aureofaciens* BS1393 cells was 10^{-6} per donor cell. The plasmid was relatively stable in the transconjugant strain maintained in LB medium under nonselective conditions (about 90% of cells retained their plasmids after 10 generations). The plasmid imparted to the recipient strain resistance to cobalt and nickel with an MIC of 8 mM in LB medium. The resistance of the transconjugants to zinc and cadmium (the minimal inhibitory concentration is equal to 4 and 0.5–0.7 mM, respectively) did not differ from that of the recipient strain BS1393 and the donor strain *Pseudomonas* sp. BS501(pBS501) (Table 1).

The Effect of Heavy Metals on the Synthesis of Phenazine Antibiotics

It is known that the recipient strain *P. aureofaciens* BS1393 synthesizes P1CA and its hydroxy derivatives,

Table 2. Influence of heavy metals on pigment production in the strains *P. aureofaciens* BS1393 and *P. aureofaciens* BS1393(pBS501)

Strain	Heavy metal and its concentration	Medium	Colony color under illumination at 366 nm	Colony color in reaction with formalin
BS1393	–	LB	Orange	Violet
BS1393 (pBS501)	–	LB	Orange	Violet
BS1393	Zn 3 mM	LB	Yellow-green	Violet
BS1393 (pBS501)	Zn 3 mM	LB	Orange	Violet
BS1393	Ni 2 mM	LB	Yellow-green	Green
BS1393 (pBS501)	Ni 2 mM	LB	Dark yellow	Violet
BS1393	Ni 1 mM	TM	Green	Green
BS1393 (pBS501)	Ni 1 mM	TM	Dark yellow	Violet
BS1393 (pBS501)	Ni 3 mM	LB	Dark yellow	FC
BS1393	Cd 0.5 mM	TM	Light yellow	Dark yellow
BS1393 (pBS501)	Cd 0.5 mM	TM	Dark yellow	Dark yellow
BS1393	Co 1 mM	LB	Dark yellow	FC
BS1393 (pBS501)	Co 2 mM	LB	Dark yellow	FC

Note: FC stands for "faintly colored."

2HP1CA and 2HP [5]. When grown on complete nutrient media, the colonies of strain BS1393 produce hydroxyphenazines and turn orange under illumination at 366 nm. The hydroxyphenazines present in cells and in the cultivation medium give a specific violet color in reaction with formalin [13]. We used this reagent to detect qualitative changes in the synthesis of phenazine antibiotics by the recipient strain BS1393 and its transconjugant BS1393(pBS501) in the presence of cobalt, nickel, zinc, and cadmium ions (Table 2). Increasing concentrations of zinc and nickel cations in the medium considerably affected the pigmentation of BS1393 colonies: at subinhibitory concentrations of these cations, the qualitative reaction with formalin was negative (Table 1). At the same time, these concentrations of cations did not affect the synthesis of phenazine antibiotics in the transconjugant BS1393(pBS501), as judged from the intense pigmentation of the colonies of this strain and the positive reaction with formalin. The presence of 0.5 mM cadmium in TM medium suppressed the synthesis of phenazine antibiotics in both strains. An elevated concentration of cadmium in the medium also diminished the pigmentation of BS1393(pBS501) colonies. The qualitative reaction with formalin in the presence of cobalt ions was indistinct (Table 2).

TLC analysis showed that the presence of 2 mM cobalt and nickel in the medium insignificantly changed the synthesis of phenazine-1-carboxylic acid and its hydroxy derivatives by the heavy metal–resistant strain BS1393(pBS501), but considerably inhibited the synthesis of these antibiotics in the heavy metal–sensitive strain BS1393. Zinc at a concentration of 4 mM was ineffective, whereas 0.5 mM cadmium completely suppressed the synthesis of phenazine antibiotics in both strains.

Analysis by HPLC showed that the recipient strain *P. aureofaciens* BS1393 grown in medium without heavy metals produced 2.63 μg P1CA per mg dry cells. The transconjugant *P. aureofaciens* BS1393(pBS501) grown in the presence of 2 mM cobalt or nickel produced 0.016 and 0.04 μg P1CA per mg dry cells, respectively, i.e., 164 and 66 times less than in the absence of these heavy metals. By comparison, the production of P1CA in the heavy metal–resistant strain BS1393(pBS501) in the presence of 2 mM cobalt or nickel was 0.02 μg/mg dry cells, i.e., decreased by only 36 times.

Fungicidal Activity

Various strains of the phytopathogenic fungus *Gaeumannomyces graminis* var. *tritici* are resistant to cobalt and nickel at concentrations up to 4 mM. Unlike the heavy metal–sensitive recipient strain BS1393, the heavy metal–resistant transconjugant strain BS1393(pBS501) suppressed the growth of the indicator strain Ggt 1818 when grown on Kanner medium with 0.5 mM cobalt or nickel (Figs. 1a, 1b). Cadmium at this concentration inhibited the growth of both bacterial strains and the indicator Ggt strain.

Accumulation of Cobalt by BS1393(pBS501) Cells

Atomic absorption spectroscopy showed that cells of the transconjugant strain BS1393(pBS501) and the donor strain *Pseudomonas* sp. BS501(pBS501) grown in the medium with 0.25 mM Co^{2+} accumulated 1.2 and

0.2 μg Co/mg protein, respectively. The amount of Co accumulated by cells of these two strains increased proportionally to its concentration in the medium between 0.25 and 1 mM and did not change with further increase in the cobalt concentration up to 1.5 and 2 mM. By comparison, the donor strain BS501(pBS501) accumulated 1.6 and 1.5 μg Co/mg protein at cobalt concentrations close to inhibitory (1.5 and 2 mM), whereas the transconjugant strain BS1393(pBS501) accumulated in this case 5.5 μg Co/mg protein (Figs. 2a, 2b).

A Comparative Electron Microscopic Study of Heavy Metal–Sensitive and Resistant Cells

The electron microscopic examination of the thin sections of cells of the heavy metal–sensitive strain BS1393 and the two heavy metal–resistant strains, BS501(pBS501) and BS1393(pBS501), showed that BS1393(pBS501) cells grown in TM medium with 2 mM Co^{2+} had single large or multiple small electron-opaque granules, which were located on the outer surface of the outer membrane and presumably contained cobalt (Fig. 3d), while BS501(pBS501) cells exhibited the presence of cobalt-containing acicular crystallites (Fig. 3c).

Multiple electron-opaque cobalt-containing structures were also detected in the intercellular space of the heavy metal–resistant strains grown in the presence of cobalt. In the control variants grown in the medium without cobalt, such structures were not detected (Fig. 3a). In the heavy metal–sensitive BS1393 cells incubated at 30°C in TM medium with glucose and 2 mM cobalt for 18 h, electron-opaque granules were also detected in the cytoplasm and in the periplasmic space of partially degraded cells (Fig. 3b), indicating that the membranes of heavy metal–sensitive cells may be permeable to cobalt cations.

DISCUSSION

A beneficial effect from the application of PGPR could be expected only if these rhizobacteria were able to actively colonize the plant rhizosphere and to compete successfully with indigenous microflora in this econiche. Uncontrolled factors, such as contamination of the environment with heavy metals, may reduce to zero the beneficial effect of PGPR as biocontrol agents of plant diseases, since phytopathogenic fungi are more resistant to heavy metals than rhizosphere pseudomonads. The transconjugant

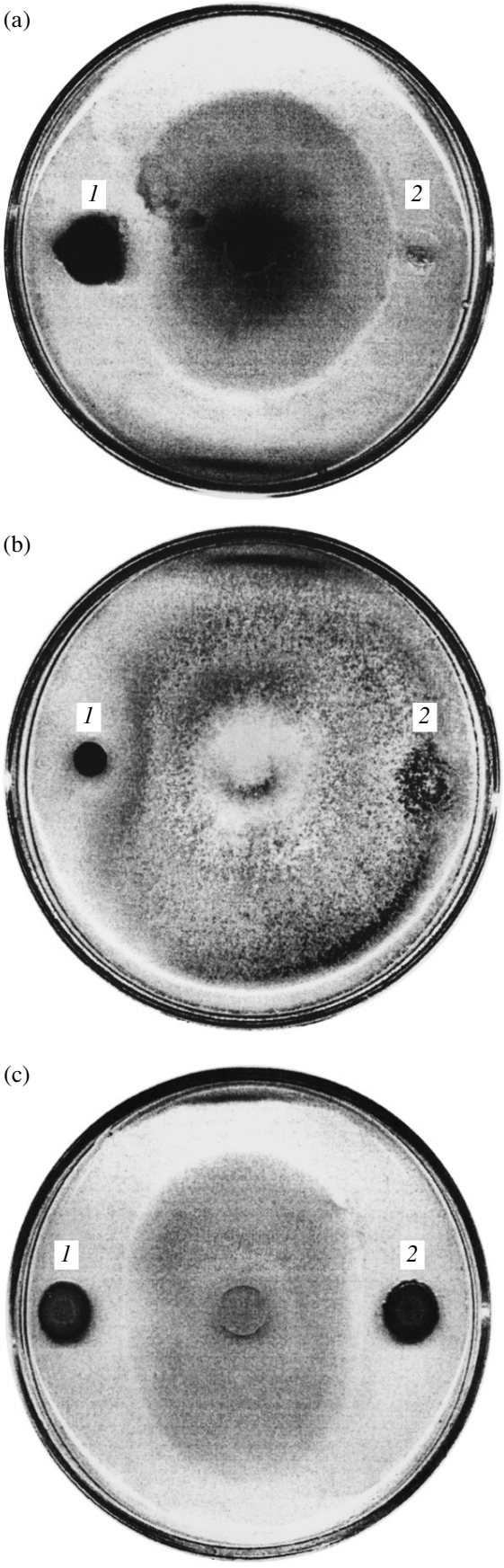

Fig. 1. The effect of (*1*) *P. aureofaciens* BS1393(pBS501) and (*2*) *P. aureofaciens* BS1393 on the growth of the phytopathogenic fungus *Gaeumannomyces graminis* var. *tritici* 1818 on Kanner agar in the presence of (a) 0.5 mM cobalt and (b) 0.5 mM nickel and (c) in the absence of heavy metals.

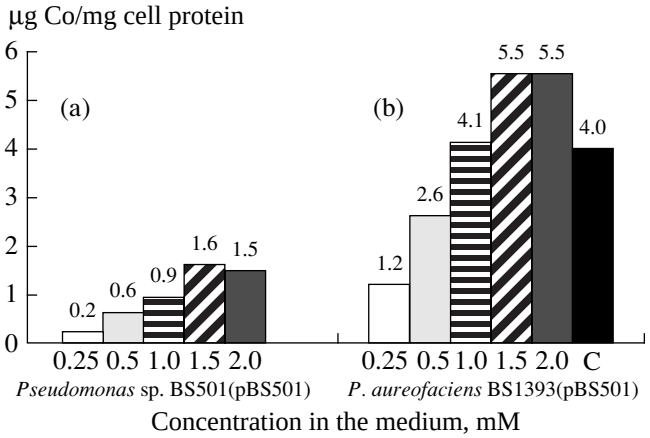

Fig. 2. The accumulation of cobalt by cells of the donor *Pseudomonas* sp. BS501(pBS501) and the transconjugant *P. aureofaciens* BS1393(pBS501) strains. C (control) shows the accumulation of cobalt by cells of strain BS1393(pBS501) grown in TM medium, washed, and incubated in the presence of 5 mM Co^{2+} at 0°C for 2 h.

P. aureofaciens BS1393(pBS501) that we derived acquired the desired properties of the donor strain *Pseudomonas* sp. BS501(pBS501) (its resistance to cobalt and nickel cations) and retained the favorable properties of the recipient strain *P. aureofaciens* BS1393 (its ability to suppress the growth of phytopathogenic fungi) (Figs. 1a–1c).

The survival of microorganisms at high concentrations of heavy metals is provided by the conversion of heavy metal cations into an inactive state or by their active excretion from cells. Some microbial exometabolites can nonspecifically fix metal ions, forming insoluble or slightly soluble phosphates, sulfates, sulfides, and other salts [14, 15]. However, heavy metals can also specifically bind to the cell surface and accumulate in cells [14–18]. The resistance of microorganisms to cobalt, nickel, zinc, and cadmium cations is usually controlled by plasmids and is provided by the energy-dependent excretion of these cations with the involvement of specific membrane protein complexes [18, 19]. The exponential pattern of cobalt accumulation was characteristic of not only the donor strain BS501(pBS501), but also the transconjugant strain BS1393(pBS501) (Figs. 2a, 2b), although the latter accumulated 4–6 times more cobalt than the former at equal concentrations of cobalt cations in the medium. The transconjugant BS1393(pBS501) cells incubated at 0°C in the presence of 5 mM cobalt or grown in the presence of 1 mM cobalt accumulated equal amounts of this heavy metal (4 μg Co/mg protein) (Fig. 2b). However, when grown at subinhibitory concentrations of cobalt (1.5 and 2 mM), BS1393(pBS501) cells accumulated more cobalt than in the case of incubation at 0°C with 5 mM cobalt. It can be hypothesized that growth at subinhibitory concentrations of cobalt is accompanied not only by the active excretion of this metal from cells, but also by the binding of cobalt cations to exometabolites, the resultant poorly soluble complexes being removed during cell washing by centrifugation.

This suggestion is confirmed by the electron microscopic observation of multiple electron-opaque structures in the intercellular space, which indicates that they are weakly bound to the cell surface. In addition, electron microscopy revealed electron-opaque granules on the outer surface of the outer membrane of BS1393(pBS501) cells, while crystallites were observed in the case of BS501(pBS501) cells. In the heavy metal–sensitive strain BS1393, electron-opaque granules were revealed not only on the cell surface, but also in the periplasmic space and in the cytoplasm of partially degraded cells, indicating that the membranes of heavy metal–sensitive cells may be permeable to cobalt cations.

The accumulation of cobalt cations on the surface of heavy metal–resistant cells, but not in their cytoplasm, implies that cobalt likely insignificantly affects the synthesis of phenazine antibiotics and fungicidal activity in the plasmid-bearing strain BS1393(pBS501). The observed decrease in the amount of P1CA in the culture liquid of the strain BS1393(pBS501) grown in the absence of cobalt may be due to a poor release of this antibiotic from cells. Earlier, we showed that the transfer of plasmid pBS501 to the *P. putida* strain BS394 was associated with the appearance of additional hydrophobic proteins and modification of the surface electric and ion-exchange properties of transconjugant cells [4]. Such a modification of the cell surface may also take place in the strain BS1393(pBS501). If so, this may impede the transport of P1CA from cells. The effect of nickel on the physiological and biochemical properties of bacterial cells must be similar to that of cobalt due to the physicochemical similarity of these heavy metals. The presence of the plasmid in the transconjugant BS1393(pBS501) provides for the survival of this strain at high cobalt and nickel concentrations. In this case, the accumulation of cobalt by cells diminishes its concentration in the medium.

An important problem in the creation of genetically engineered microorganisms for the purpose of phytobioremediation of heavily polluted soils is to combine the genetic systems responsible for heavy-metal resistance, antiphytopathogenic activity, the degradation of polycyclic aromatic compounds, and the stimulation of plant growth.

When occurring in one bacterial cell, these systems may interact. For instance, the transfer of plasmid pBS216, responsible for naphthalene biodegradation, to the PGPR *P. putida* BS1380 was found to enhance the synthesis of the phytohormone indole-3-acetic acid in the transconjugant [20]. Similarly, changes in the synthesis of phenazine antibiotics observed for the

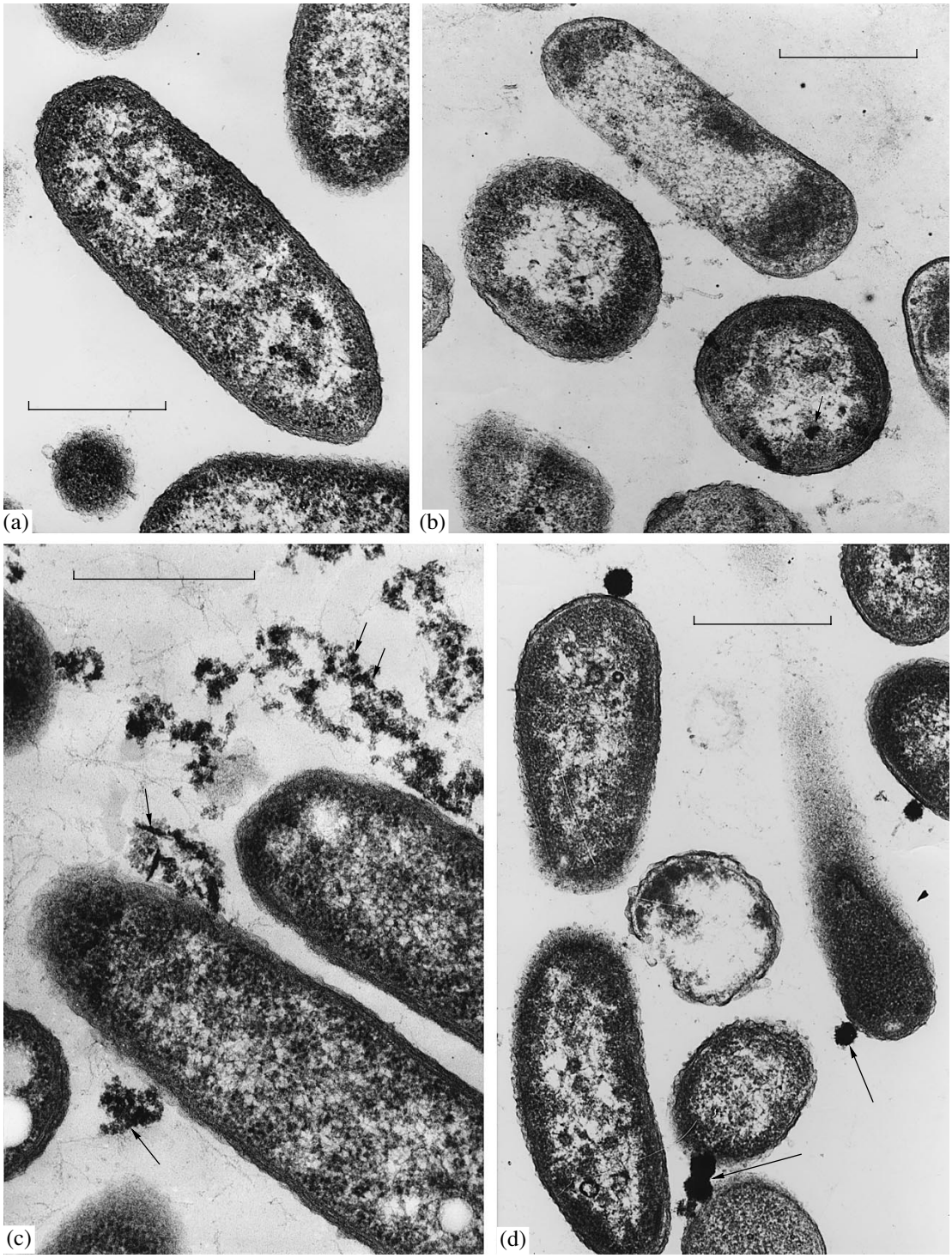

Fig. 3. Electron microscopy of (b) *P. aureofaciens* BS1393, (c) *Pseudomonas* sp. BS501(pBS501), and (d) *P. aureofaciens* BS1393(pBS501) cells grown in the presence of 2 mM $CoCl_2$. Panel (a) shows *P. aureofaciens* BS1393 cells grown in the absence of heavy metals. Bar, 0.5 μm.

transconjugant *P. aureofaciens* BS1393(pBS501) may be associated with the interaction of the genetic systems responsible for heavy-metal resistance and the synthesis of phenazines.

Thus, the results presented clearly demonstrate the possibility of obtaining genetically modified heavy metal–resistant PGPR and applying them in agriculture for the bioremediation of soils contaminated with heavy metals.

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