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EXPERIMENTAL ARTICLES

Isolation and Characterization of a Unicellular Manganese-Oxidizing Bacterium from a Freshwater Lake in Northwestern Russia

A. A. Falamin and A. V. Pinevich[1]

Biological Research Institute, St. Petersburg State University, Oranienbaumskoe sh. 2, Staryi Petergof, St. Petersburg, 198904 Russia

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Abstract—A unicellular manganese-oxidizing bacterium (strain L7), isolated from Lake Ladoga, is identified as *"Siderocapsa"* sp. according to its morphology. However, this bacterium belongs to the phylogenetic cluster of *Pseudomonas putida*. The physiological characteristics (utilization of sugars, polyols, organic acids, and phenolic substrates as carbon and energy sources) also indicate the similarity of strain L7 to representatives of the genus *Pseudomonas*. The growing culture oxidizes Mn(II); the rate of oxidation depends on the type of added organic substrate. Carbonate requirement for this process indicates mixotrophic metabolism. The relatedness of the isolated bacterium to the representatives of the genus *Pseudomonas* and their phenotypic similarity provide a basis for considering strain L7 not as *"Siderocapsa"* sp., but as a new species, *Pseudomonas siderocapsa* sp. nov., of the *P. putida* cluster.

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The natural cycle of manganese is under the control of prokaryotes. Mobile manganese compounds act as shuttles that connect aerobic and anaerobic ecosystems [1, 2]. In the aerobic ecosystems, the process of oxidation of bivalent manganese to dioxide dominates; manganese oxidizers use this reaction for protection against reactive oxygen species and, possibly, for bioenergetic purposes. One of the results of Mn^{2+} cation oxidation is its detoxication, which gives a selective advantage in manganese-rich environments. Manganese dioxide is often accumulated in microbial cell envelopes [3]. Oxidized manganese penetrates into the sediments, where it can be used as an electron acceptor for anaerobic respiration. Reduction of Mn(IV) occurs simultaneously with dissimilatory sulfate and nitrate reduction and with methanogenesis. Similar processes occur in flooded soils, and Mn(II) returns to aerobic ecosystems [4].

The metabolic role of the manganese transformations performed by bacteria is the main issue of the microbiology of this element [3]. Organisms capable of manganese lithotrophy and manganese chemosynthesis are of great interest, but their existence has not been strictly proven yet.

The representatives of the genus *"Siderocapsa"*, which form capsules where manganese oxides accumulate, are the least investigated among the Mn(II) oxidizers. Although they have been repeatedly detected in marine and freshwater ecosystems, they were not isolated in pure cultures. Due to the absence of a physiological and biochemical diagnosis, the validity of this taxon can be called into question (see [5]).

A pure culture of manganese-oxidizing bacterium became available only recently [6]. The goal of the present work was to determine its phylogenetic position and its phenotypic properties. Attention was focused on Mn(II) oxidation.

MATERIALS AND METHODS

Source and isolation of pure cultures. The collection of manganese-oxidizing bacteria was isolated from the water of Lake Ladoga (northwestern Russia), sampled from the decline zone. The water temperature was about 13°C. Samples were inoculated into BG-11 medium (see below), with 4% agarose (Difco), and incubated at 30°C for at least a weak. The surface colonies, colored with manganese oxides, were purified by repeated transfers. Twelve strains (L1–L12) were isolated. Strain L7, which grew most actively in liquid medium BG-11, was selected for further investigations.

Conditions of cultivation. The modified medium BG-11 was used [7], contained (mg l^{-1}): $NaNO_3$, 300; $MgSO_4 \cdot H_2O$, 75; $K_2HPO_4 \cdot 3H_2O$, 40; $CaCl_2 \cdot 2H_2O$, 36; Na_2CO_3, 20; citric acid, 6; $Fe_2(SO_4)_3 \cdot 9H_2O$, 6;

[1]Corresponding author; e-mail: A.Pinevich@paloma.spbu.ru

NH_4Cl, 6; $Na_2MoO_4 \cdot 2H_2O$, 4; H_3BO_3, 3; $MnCl_2 \cdot 4H_2O$, 2; $ZnSO_4 \cdot 7H_2O$, 2; Na_2EDTA, 1; $CuSO_4 \cdot 5H_2O$, 0.1; $Co(NO_3)_2 \cdot 6H_2O$, 0.05 (pH 7.6). The culture was maintained on BG-11 medium at room temperature with monthly transfers. The culture was grown in 250-ml Erlenmeyer flasks; the inoculum dose was 5%. The cultivation was performed at a temperature of 30°C for seven days, except when otherwise specified.

Electron microscopy. The material was collected by centrifugation (1500 g, 5 min) and was washed with 100 mM Na-cacodylate buffer (pH 7.2). Prefixing was performed with 2% glutaraldehyde in cacodylate buffer at 6°C for 2 h, and fixation was accomplished with 1% OsO_4 in the same buffer at 5°C for 12 h. The fixed material was immobilized in 2% agar (Difco) and dehydrated in ascending concentrations of ethanol, with a mixture of absolute ethanol with absolute acetone (1:1), and then in absolute acetone. The material was transferred to a Spurr mixture with acetone, then to the Spurr mixture without acetone, and was polymerized at 65°C for 24 h. Bacterial ultrathin sections with a thickness of 60–100 nm were made using Reichert ultratome; they were placed on formvar-coated copper grids and contrasted according to Reinolds. The preparations were examined under a Tesla BS-500 electron microscope with a working magnification of 30000.

Phylogenetic analysis. The chromosomal DNA preparation was obtained according to a standard protocol [8]. DNA was used for PCR amplification with universal primers for bacterial 16S rRNA gene:

fD1: 5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3' (forward primer);

rP1: 5'-CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT-3' (reverse primer).

PCR was performed in 50 μl of the buffer (10 mM Tris–HCl, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2.5 mM $MgSO_4$) containing 10 ng of DNA, 0.4 U of *Pwo* DNA polymerase, 20 pmol primers, and 10 nmol dNTP. The PCR product with a length of about 1500 bp was extracted from agarose gel and inserted into the cloning vector pTOPO 2.1; the presence of the insertion was confirmed by analysis with *Eco*RI restriction endonuclease. The PCR product was sequenced in three steps, about 600-bp each; the two terminal parts and the medium part partially overlapped. The reconstructed sequence of the 16S rRNA gene was compared with other bacterial sequences using BLAST.

Quantitative methods. Bacterial growth was assayed by protein concentrations with the Lowry method. The Mn(II)-oxidizing activity was determined by the persulfate method, according to formation and accumulation of manganese oxides in the capsule [9]. To determine the optimal growth temperature, bacteria were incubated in BG-11 medium at 20–35°C. Optimal pH was determined using a BG-11 medium supplemented with 5 mM phosphate buffer (pH 5.8–8.0); pH values of the media were determined at the beginning and at the end of the experiment. For glucose determination, glucose oxidase method was used [10].

Research of the lithoautotrophic metabolism. To investigate dependence of the growth and of manganese-oxidizing activity on Mn(II) concentration, the culture was grown on a BG-11 medium supplemented with 10–700 μM Mn(II). For investigation of carbonate influence, the following variants of BG-11 medium were used: without carbonate; containing the standard carbonate concentration (0.2 mM); containing tenfold carbonate concentration; and containing tenfold carbonate concentration and twentyfold concentration of Mn(II) (200 μM).

Research of the heterotrophic metabolism. To investigate the capability to utilize organic substrates, the culture was grown in a BG-11 medium containing (1 mM): arabinose, sodium acetate, glucose, inositol, xylose, lactose, maltose, rhamnose, ribose, sucrose, sorbitol, fructose, fumaric acid, or sodium citrate. To determine the capability to oxidize recalcitrant substrates, *o*- and *p*-phenylenediamine (1 mM), and N,N'-dimethyl-*p*-phenylenediamine (0.01 of the saturating concentration) were used.

Statistical analysis of data. The experiments were performed in three replicates; the mean values are presented. For statistical analysis, Student's criterion for $P = 0.95$ was used.

RESULTS AND DISCUSSION

Isolation and cultivation. After inoculation of water samples into the BG-11 medium (commonly used for the isolation of cyanobacteria), brown colonies were observed. At first, this finding was attributed to photosynthetic pigments. However, in the absorption spectrum in the 650–1100 nm range, no peaks of chlorophyll or bacteriochlorophyll were found; this result demonstrated that the bacterium did not belong to phototrophs or quasiphototrophs. Since the growth of strain L7 was independent of light, an assumption was made that this bacterium was a chemolithotrophic organism and utilized the Mn(II) present in the BG-11 medium.

Morphology and ultrastructure. In liquid medium, the culture formed a loose ocher sediment which gradually condensed and darkened. On solid medium, opaque, flat colonies with an even edge appeared. A young culture consisted of short motile rods ($1.11 \pm 0.48 \times 0.78 \pm 0.14$ μm); in the stationary phase of growth, it consisted of coccobacilli with an average size of 0.5–0.7 μm. On micrographs, the following structures are visible: a capsule containing manganese oxides; a cell wall of the gram-negative type; a peripheral cytoplasm zone filled with ribosomes; the nucleoid central region; and electron-dense polyhedral inclusions (Fig. 1). These inclusions resemble magnetosomes, but the cells do not respond to a magnetic field. Most probably, these inclusions are of the same type as the microcrystals of *Shewanella putrefaciens*

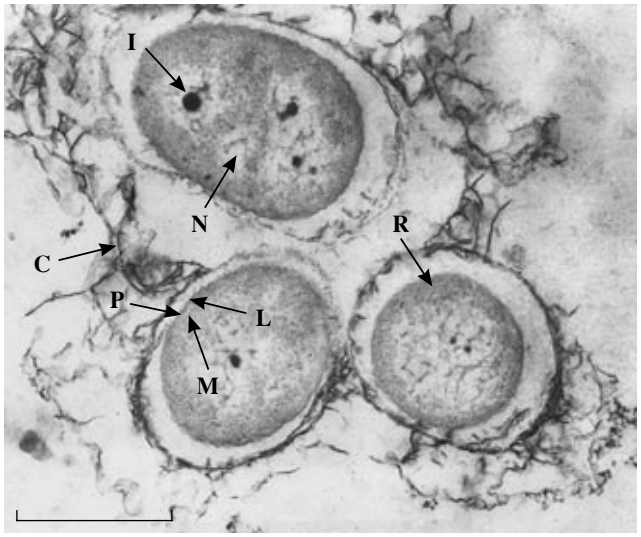

Fig. 1. Electron micrograph of a thin section of a group of strain L7 cells in a common capsule: I, inclusion; C, capsule; L, lipopolysaccharide membrane; M, cytoplasmic membrane; N, nucleoid; P, peptidoglycan layer; R, ribosomes. Scale bar, 0.5 μm.

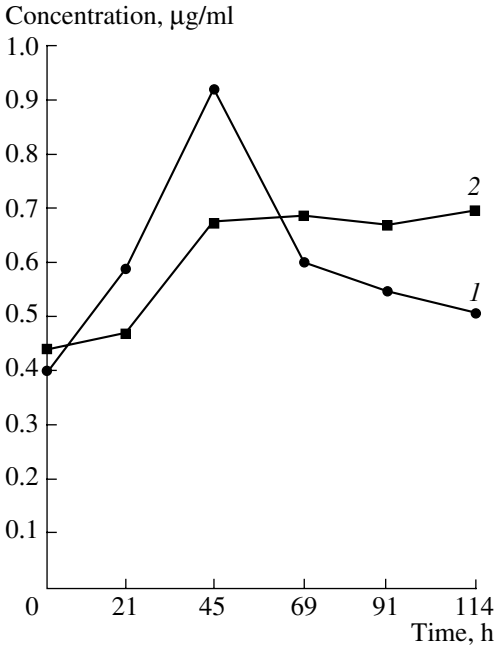

Fig. 2. Cell protein content (*1*) and manganese oxidation (*2*) in a growing culture of strain L7.

CN32, which are formed during dissimilatory reduction of ferrihydrite and are used for the accumulation of iron in a nontoxic form [11].

The presence in the capsule of manganese with an oxidation level ≥ +3 was demonstrated by the positive reaction with leucoberbelin blue; this reaction was performed according to the standard procedure (see [12]). Thus, the biogenic mineral is either a Mn(III) oxide, a Mn(IV) oxide, or a mixture of the two. Complexes of Mn(III) with organic chelating agents are known to be the products of fungal laccase (homologous to the representatives of the superfamily of Mn(II) oxidases) [3, 13]. Formation of manganese dioxide in the exosporium of *Bacillus* sp. SG-1 passes the stage of Mn(III) formation [1]. Strain L7, apparently, also initially generates Mn(III), which is then disproportionated to Mn(II) and insoluble dioxide. This may be the reason for the change from the initial ocher color of the precipitate to brown.

These characteristics (gram-negative morphotype, formation of capsule, and Mn(II) oxidation) allow strain L7 to be assigned to the genus *"Siderocapsa"* (see [5]).

Phylogenetic relations. As a result of amplification and sequencing of the segment of chromosomal DNA encoding 16S rRNA, the complete nucleotide sequence (1532 bp) of the corresponding gene was determined. According to the data obtained, strain L7 was most close (>99%) to the strains *Pseudomonas* sp. IpA-2 and DhA-51, which utilize recalcitrant substrates and possibly exhibit Mn(II)-oxidative activity. Of the validly described *Pseudomonas* species, *P. jessenii* and *P. putida* are the nearest relatives of strain L7 (99% homology).

Thus, strain L7, as most Mn(II)-oxidizing bacteria, belongs to phylum BX *Proteobacteria*, class *Gammaproteobacteria*, gene cluster of *P. putida*. This conforms to the capability of other pseudomonads to oxidize Mn(II); for example, the *P. putida* cluster contains Mn(II)-oxidizing strains MnB1 and GB-1 [3, 14].

Dynamics of bacterial growth and its dependence on the physical and chemical parameters of the medium. Strain L7 does not exhibit an exponential stage of growth. This phenomenon is rare among bacteria. Protein is accumulated over 45 h and manganese is oxidized in the same period; afterwards the culture enters a stationary phase (Fig. 2).

Growth dependence on temperature (Fig. 3) is a curve with an optimum at 25–30°C. The permissive area is apparently wider than the tested interval (20–35°C). Thus, this bacterium belongs to mesophiles; this finding agrees with the data obtained for Mn(II)-oxidizing bacteria of the genera *Leptothrix* and *Pedomicrobium* and for the marine α-proteobacterium SD-21 [15, 16]. The only known manganese-oxidizing thermophile is *Caldimonas manganoxidans*, with the growth optimum at 50°C [17].

Attempts to determine the effect of pH on growth were undertaken in the pH range of 5.8–8.8 and did not reveal an optimum. This fact allows us to describe this bacterium as a neutrophile with a wide range of pH tolerance. Most Mn(II)-oxidizing bacteria also grow at pH 5.8–8.0, but they have a pH optimum at about 7.0–8.0 [16, 17]. Identical growth in this pH range does not

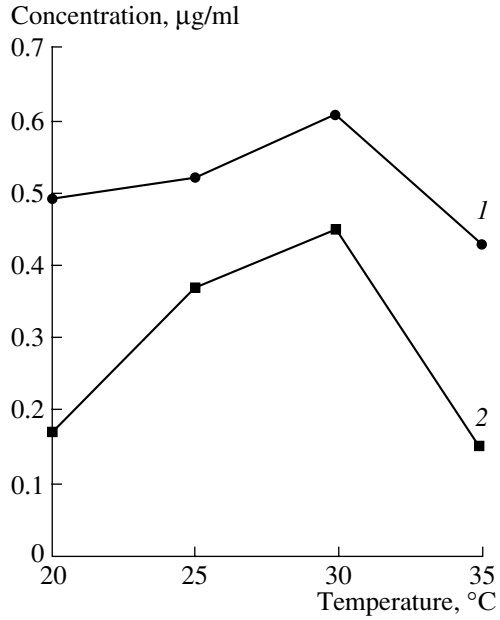

Fig. 3. Cell protein content (*1*) and manganese oxidation (*2*) in the culture of strain L7 under different temperatures.

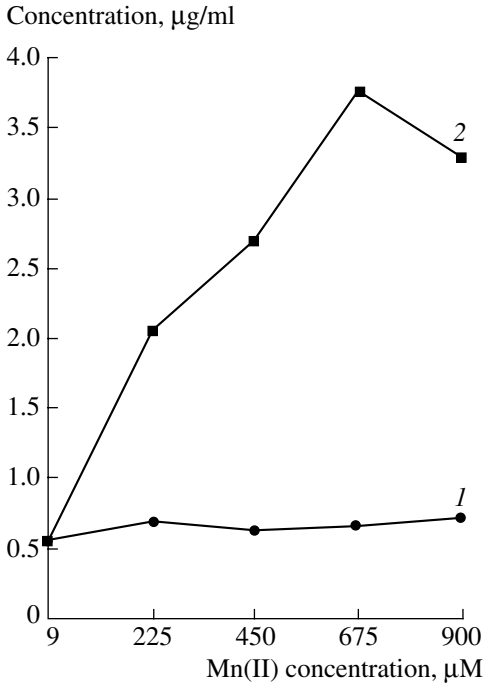

Fig. 4. Cell protein content (*1*) and manganese oxidation (*2*) in the cultures of strain L7 in the medium supplemented with different concentrations of manganese.

imply the absence of a pH optimum for Mn(II) oxidase. The absence of an evident reaction to pH may be caused by another limiting factor that does not allow the enzymatic activity to increase, even at the optimal pH value. The carbon and/or energy source most probably acts as this factor.

Lithoautotrophic metabolism. Manganese is the main source of energy in the BG-11 medium. Dependence of the Mn(II)-oxidizing activity on Mn(II) content has the shape of a saturation curve (Fig. 4). An increase in the manganese concentration stimulates accumulation of its oxides; i.e., the standard medium BG-11 (10 μM Mn^{2+}) is limited in the energy source. However, the biomass value was equal to the control because of the low concentration of carbonate, which served as the carbon source for autotrophic growth.

The intensification of the lithoautotrophic metabolism of strain L7 by simultaneous increase of Mn(II) and carbonate concentrations was unfeasible for two reasons. First, since $MnCO_3$ solubility is 9 μM, a considerable portion of the manganese precipitates even in the presence of chelating agents. Second, carbonate shifts the medium pH to alkaline values and, therefore, destabilizes manganese and stimulates its chemical oxidation. Nevertheless, twentyfold increased concentration of Mn(II) and tenfold increased concentration of carbonate resulted in a twofold increase in bacterial biomass (by protein) compared to the control (from 0.44 ± 0.07 μg/ml to 1.00 ± 0.12 μg/ml).

Mn(II) oxidation concomitant with biomass accumulation indicated lithotrophic growth with an inorganic carbon source. To prove this assumption, carbonate was excluded from the medium. An insignificant increase of biomass was observed, but manganese was not oxidized. Carbonate is possibly required for two reasons: this substrate is used for autotrophic growth and it also binds Mn^{2+} cations, decreasing the energy of activation in the oxidase reaction (see [18]).

Heterotrophic metabolism. Only heterotrophic utilization of chelating agents (citrate or EDTA) can explain the weak growth observed in the medium without carbonate. To investigate the capability of strain L7 to metabolize organic acids, acetate, fumarate, or citrate (1 mM) were added to the medium. In all cases, the biomass increased considerably compared to the control. The amount of manganese dioxide formed depended on the substrate: in the medium with fumarate, it was equal to the control; with acetate, it was higher, and with citrate, lower than in the control (table).

Content of the cell protein (μg/ml) and manganese oxidized (μg/ml) in 7-day cultures of strain L7 grown in media with organic acids ($M \pm m$, $n = 4$)

Variant of the experiment	Protein	Manganese oxidized
Control	0.33 ± 0.03	0.36 ± 0.08
Acetate	1.52 ± 0.17	0.76 ± 0.12
Fumarate	0.94 ± 0.14	0.40 ± 0.10
Citrate	2.38 ± 0.19	0.20 ± 0.04

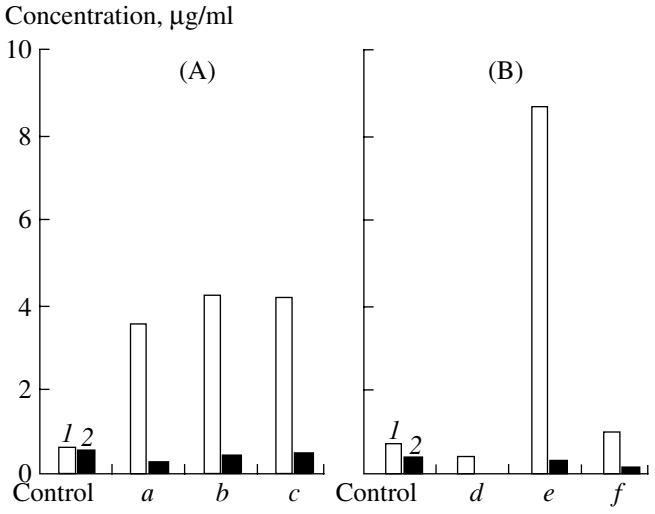

Fig. 5. Cell protein content (*1*) and manganese oxidation (*2*) in the cultures of strain L7 grown in media supplemented with sugars (A) and phenylenediamines (B); (*a*) glucose; (*b*) fructose; (*c*) sucrose; (*d*) *o*-phenylenediamine; (*e*) *p*-phenylenediamine; (*f*) N,N'–dimethyl–*p*–phenylenediamine.

Most of the bacteria which oxidize cations of metals are capable of mixotrophic growth. For example, *Gallionella ferruginea*, which was previously considered an autotroph, can oxidize Fe^{2+} simultaneously with glucose, sucrose, or fructose [19].

Bacterial biomass was almost an order of magnitude higher than in the control during the growth in BG-11 media supplemented with glucose, sucrose, or fructose (Fig. 5A). As Mn(II)-oxidizing activity was not higher than in the control, the metabolic balance certainly shifted to heterotrophic metabolism. A similar effect was observed in cultures of *G. ferruginea* in the medium supplemented with 20 μM glucose, when assimilation of carbon dioxide in the Calvin cycle was simultaneously reduced [19]. Strain L7 is more capable of heterotrophic growth than *G. ferruginea*, which does not utilize organic acids [20]. Apart from the substrates listed above, strain L7 utilized arabinose, inositol, xylose, lactose, maltose, rhamnose, ribose, and sorbitol (1 mM). In all these cases, the biomass was almost fivefold higher than in the control, and Mn(II)-oxidizing activity was somewhat lower than in the control. On the medium with glucose, the culture exhibited a pronounced exponential growth phase (16–44 h); during this period, all glucose was consumed. Manganese was oxidized simultaneously; this finding also indicated mixotrophic metabolism.

Recalcitrant substrates oxidation. Many bacteria of the genus *Pseudomonas* are known to utilize recalcitrant substrates. For example, Mn(II)-oxidizing pseudomonads oxidize *p*-phenylenediamine and ABTS (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate)). Utilization of aromatic substrates is typical for *P. putida*, closely related to strain L7 [21].

In the medium with *o*-phenylenediamine, Mn(II) was not oxidized at all and the biomass was lower than in the control. This fact points to the inhibitory properties of this compound. In the presence of N,N'-dimethyl-*p*-phenylenediamine, growth was slightly higher, while manganese dioxide accumulation was lower then in the control. However, the biomass yield in the medium with *p*-phenylenediamine was an order of magnitude higher than in the control; it was even twofold higher than in the medium with carbohydrates. The concentration of manganese dioxide was at the control level (Fig. 5B).

Growth in the medium with phenylenediamines indicates the physiological similarity of strain L7 to Mn(II)-oxidizing strains of the *P. putida* cluster. Since growth with *p*-phenylenediamine was better than with carbohydrates or organic acids, in nature, strain L7 probably specializes in the oxidation of recalcitrant substrates.

Phenylenediamine oxidation is regiospecific. Mn(II) oxidase probably participates in this process, while manganese dioxide or the complex of Mn(III) with organic acids most probably act as oxidizing reagents. Their interaction with aromatic compounds must cleave the benzene ring with the formation of simpler molecules; the mutual position of substitutes is important. As *o*-phenylenediamine inhibits bacterial growth and *p*-phenylenediamine stimulates it, the carbon atom between the two amine groups of the benzene ring is probably oxidized. The weaker effect of N,N'-dimethyl-*p*-phenylenediamine is apparently the result of the partial screening of the target atom by the methyl radicals bound with amino groups. In this case, manganese acts not as an energetic substrate, but as a reagent for the destruction of aromatic compounds; this function broadens the biologic role of manganese oxidation.

Thus, a freshwater unicellular Mn(II)-oxidizing bacterium satisfying the canonical description of the genus *"Siderocapsa"* was isolated in pure culture. It was found to be related to the *P. putida* phylogenetic cluster and to have a metabolism of the mixotrophic type. Further research will make it possible to specify its metabolic features, in particular, to investigate the interaction between manganese oxidase and the respiration chain.

These results allow us to revise the original identification of strain L7 as *"Siderocapsa"* sp. and to assign it to the genus *Pseudomonas*. Like other pseudomonad species, this bacterium utilizes a range of organic substrates, including recalcitrant ones. At the same time, it possesses the characteristics not previously described for pseudomonads: a capsule accumulating manganese oxides and a capability for mixotrophic growth (with manganese as an additional source of energy, even in the presence of organic substrates). Unlike other Mn(II)-oxidizing pseudomonads, strain L7 accumulates manganese oxides not on the outer membrane [3]

but in the capsule. This characteristic alone, however, is insufficient for the preservation of the genus *"Siderocapsa"*.

The family "*Siderocapsaceae*" was described in the middle of the 20th century to comprise iron- and manganese-oxidizing unicellular bacteria without appendages and not forming rosettes. However, the lack of specific physiological data made this definition not sufficiently valid. We share the point of view that *"Siderocapsa"* spp. are in fact poorly investigated morphological variants of bacteria of other species [5]; taking into account the results of the present work, we believe it possible to refrain from the further use of this genus.

Based on the genetic relatedness of strain L7 and on its high physiological similarity to pseudomonads, we suggest considering it a member of the new species *Pseudomonas siderocapsa* sp. nov. in the gene cluster of *P. putida*. The main diagnostic features of *P. siderocapsa* are the typical pseudomonad morphology; the presence of a capsule containing manganese dioxides; Mn-oxidizing activity; and mixotrophic type of metabolism.

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