Revista Latinoamericana de Microbiología (1998) 40:152-157
Asociación Latinoamericana de Microbiología

Identification of Manganese-Oxidizing *Bacillus* spp. from Microbial Mats at Baja California, México

Alejandro López-Cortés* and Margarita González del Pliego

Universidad Nacional Autónoma de México (UNAM), Facultad de Medicina, México, 04510, D. F.

Corresponding author. Present address: The Center for Biological Research of the Northwest (CIBNOR) La Paz, B.C.S., 23000, México. P.O. Box 128. Phone: (112) 536-33 ext 197. Fax: (112) 547-10 or (112) 536-25.
E-mail: alopez@cibnor.mx

*Corresponding author

ABSTRACT. Four endospore-forming bacterial strains which precipitate manganese oxides were isolated from laminated microbial mats at Laguna Figueroa, Baja California, Mexico. The identification of these bacteria, referred to as strains NP-36-80-L, NP-36-80-G, P2-V-83-2, and P2-V-83-3 has been established on the basis phenotypic characteristics. Three strains were identified as *Bacillus licheniformis*, and the other as *Bacillus brevis*, which are previously undescribed isolates that precipitate manganese in aging cultures.

RESUMEN. Cuatro cepas de bacterias formadoras de endosporas, que precipitan óxidos de manganeso fueron aisladas de alfombras microbianas laminadas de la Laguna Figueroa, Baja California, México. La identificación de esas bacterias, referidas como cepas NP-36-80-L, NP-36-80-G, P2-V-83-2, y P2-V-83-3 ha sido establecida con base en sus características fenotípicas. Tres de las cepas fueron identificadas como *Bacillus licheniformis* y una como *Bacillus brevis*, las cuales corresponden a aislamientos previamente no descritos, que precipitan manganeso en cultivos viejos.

INTRODUCTION

The biological significance of manganese-oxidizing bacteria has been recognized since the beginning of this century.[1] Several studies on microbial manganese oxidation show its importance in six fields: (1) In ecology for its role in the geochemical cycle of manganese, which is an important trace element in biological systems, essential in microbial, plant, and animal nutrition, and required as an activator by a number of enzymes. It is also a model to explain the genesis of nodules rich in manganese oxides, which occur on the floor of the oceans.[3] (2) In biochemistry there have been some mechanisms elucidated that explain manganese oxidation by enzymatic and non-enzymatic processes.[3, 14] (3) In genetics, the modern molecular approaches allowed the characterization of a operon in the marine *Bacillus* sp. strain SG-1 that coded for a manganese-oxidizing protein.[15] (4) In biotechnology, microbial metal precipitation has a number of industrial applications including environmental metal detoxification and clean up, waste treatment, recovery of pollutant or precious metals, production of catalysts or adsorbents, and the production of new biomaterials.[14] (5) In paleobiology, for its role of biomineralization, precipitation, and mineralization of cell envelopes of bacterial soft cells and its implication in their preservation as microfossils.[2] (6) In biodiversity to determine the taxonomic status of the microorganisms with the ability to oxidize manganese.

There are about 20 genera of manganese-oxidizing bacteria,[9] and the assignment of the taxonomic position at the species level of members of the genus *Bacillus* with the ability of precipitate manganese oxides has received little attention. Two examples studied are a non-marine strain of *Bacillus subtilis* CU1037[13] and an euryhaline strain, *Bacillus megaterium* BC1 from a microbial mat.[4]

This paper establishes the taxonomic position of four endospore-forming bacterial strains associated with manganese oxide precipitates on the surface of spores, which were isolated from laminated microbial mats in a closed hypersaline lagoon at Laguna Figueroa, Baja California, Mexico. The strains NP-36-80-L, NP-36-80-G, and P2-V-83-2 are new strains of *Bacillus licheniformis*, and the strain P2-V-83-3 is a new strain of *Bacillus brevis*, determined on the basis of their phenotypic characteristics and precipitation of manganese oxides on the surface of its spores.

MATERIAL AND METHODS

Sources, bacterial strains, isolation, and cultivation. Manganese-oxidizing bacteria were isolated from the black-brown layers of the microbial mats. Laminated microbial mats were collected from two sites ("North Pond" and "Pentapus Salina") at Laguna Figueroa, B.C., México.[6, 8] "North Pond" mats from 1-cm depth were

Table 1. Phenotypic characteristics that distinguish strains of the four isolates, *Bacillus licheniformis*, *Bacillus brevis*, and *Bacillus subtilis*

Characteristics	NP-36-80-L	NP-36-80-G	P2-V-83-2	*B. licheniformis**	P2-V-83-3	*B. brevis**	*B. subtilis* ATCC 6051e
Width of rod (µm)	0.4-0.7	0.4-0.7	0.4-0.7	0.6-0.8	0.9-1.0	0.6-0.9	0.7-0.8
Length of rod (µm)	2.5-3.0	2.5-3.0	2.5-3.0	1.5-3.0	3.5-5.0	1.5-4.0	2.0-3.0
Sporangium swollen	-	-	-	-	+	+	-
Spore shape	E	E	E	E	E	E	E
Spore position	T	T	T	C	C/T	C/T	C
Catalase	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	-	-	-
Acetoin production	+	+	+	+	-	-	+
Acid from							
D-Glucose	+	+	+	+	-	d	+
L-Arabinose	+	+	nt	+	nt	-	+
D-Xylose	+	+	nt	+	nt	-	+
D-Mannitol	+	+	nt	+	nt	d	+
Gas from glucose	f	f	f	f	-	-	-
Hydrolisis of							
Casein	+	+	+	+	-	d	+
Gelatin	d	-	-	+	-	d	+
Starch	+	+	+	+	-	d	+
Use of citrate	+	-	-	+	-	d	+
Deamination of phenylalanine	-	-	-	-	-	-	-
Nitrate reduced to nitrite	+	+	+	+	-	d	+
NaCl required	-	-	-	-	-	-	-
Growth at pH 6. 8 Nutrient broth	+	+	+	+	+	+	+
Growth at 5.7 SDA	+	+	+	+	-	d	+
Growth in NaC l7%	+	+	+	+	nt	d	+
Growth at 55°C	+	+	+	+	+	d	-

E, ellipsoidal; T, terminal to subterminal; C, central; C/T, central to terminal; d, positive between 11% to 89%; F, few bubbles; SDA, Sabouraud Dextrose Agar.

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Table 2. Qualitative determination of manganese oxide precipitates by *Bacillus licheniformis* and *Bacillus brevis* strains after 30 days of incubation.

Criteria	NP-36-80-L	NP-36-80-G	P2-V-83-2	P2-V-83-3
Brown color intensity of the coloniesin K medium without Mn (II)	-	-	-	-
Brown color intensity of the coloniesin K medium with Mn (II)	- to +	- to ++	- to ++	+++
Blue color intensity with LBB reagentin K medium with Mn (II)	- to ++	+++	+++	+++

(-), colonies without color; (- to +), colonies without color to light color; (- to ++), colonies without color to moderate color; (+++), intensive color.

placed directly into sterile Petri dishes and transported to the laboratory in an anaerobic jar using BBL 60465 CO_2+H_2 system without temperature control. Sea Water Complete Medium (SWC) formulated as follows: peptone (Difco Bacto) 5.0 g, yeast extract 3.0 g, glycerol 3.0 ml, and agar 12 g, in 1000 ml of artificial seawater[7] was used for the isolation. One Gram of the lower black layer was suspended in artificial seawater[8] and serial dilutions spread on solid SWC were made. The plates were incubated at 30 °C for 24 to 48 hours. The same suspensions were heat-treated at 75 °C for 10 min for the recovery of mesophilic endospore-forming bacteria on SWC by the same procedure. Subsamples from "Pentapus Salina" were directly placed on modified solid K medium,[8] transported to the laboratory without temperature control, and two-days later incubated in aerobic conditions at 30 °C for eight days for the isolation of manganese-oxidizing bacteria. The strains obtained from the two media were routinely grown and maintained on modified K medium.

LBB spot test for manganese dioxide. On solid medium K (1.5% agar) and for qualitative manganese determinations, oxidation was detected by spot tests using leukoberbelin blue (LBB) reagent,[5] which forms characteristic blue-colored compounds on reaction with manganese oxide. A negative control was done in K medium without a manganese source.

Transmission Electron Microscopy. Bacterial samples were prepared for transmission electron microscopy. A bacterial pellet was fixed with 3% glutaraldehyde solution in 0.15 M phosphate buffer, pH 7.3, for 6 to 8 h at 25 °C. After washing three times with the same buffer solution (30 min each), the sample was postfixed in 1% osmium tetroxide (OsO_4) with ruthenium red (0.05 %) in 0.15 M phosphate buffer, pH 7.4, for 12 hours at 25 °C. Samples were then dehydrated in ethanol (70, 80, 90, and 100%) for 30 min each concentration, and two times in 100% propylene oxide for 40 min. They were embedded in Epon 812, and the samples polymerized for 24 hours at 60 °C. Thin sections were stained with 2% uranyl acetate for 20 min, and lead citrate for 5 min,[10] and studied and photographed using a Zeiss TEM, Model EM9-S2.

Cell cycle and colony formation. Chamber microculture was made by placing 100 µl of modified liquefied K medium on a clean and sterile slide. Once, solidified 10 µl of diluted bacterial suspension is added and spread with bacterial loop. A coverslip was place on the inoculated K medium. This was then sealed with a mixture of paraffin and petrolatum to avoid contamination and keep the media moist. The microcultures were incubated in a sterile petri dish at 30 °C. Observations were made a different times with a phase contrast microscope NIKON Labophot (Japan).

Bacterial Identification. The strains isolated were identified according the colony and cellular morphology, and physiological and biochemical traits.[12] *Bacillus subtilis* e 6051 ATCC was used as a reference strain in all the tests. The characterization was done in duplicate three times or more, and the axenic condition of the strains was routinely checked.

RESULTS

Manganese-oxidizing bacteria were recovered not only from fresh mat samples but also from dried mat samples, even after several months of storage. Strong manganese oxidizers were far more prevalent in the lower black layers of the mat than they were in the upper layers. Four strains of endospore-forming bacteria that oxidize manganese were obtained as axenic cultures and identified at the species level.

An euryhaline, facultatively anaerobic bacterial strain NP-36-80-L was recoved from "North Pond" mat samples. The strain was obtained on SWC agar. Colonies were mounds and lobes consisting largely of slime. Further purification was achieved by dilution and streaking on modified K medium. Colonies become opaque with rough surface and hair-like outgrowths usually attached strongly to agar. Red pigment was formed on carbohydrate media containing sufficient iron. Aged cultures in K medium become brown. This strain was motile, Gram-positive rod-shaped cells 0.4- to 0.7-µm wide and 2.5- to 3.0-µm long. Cells

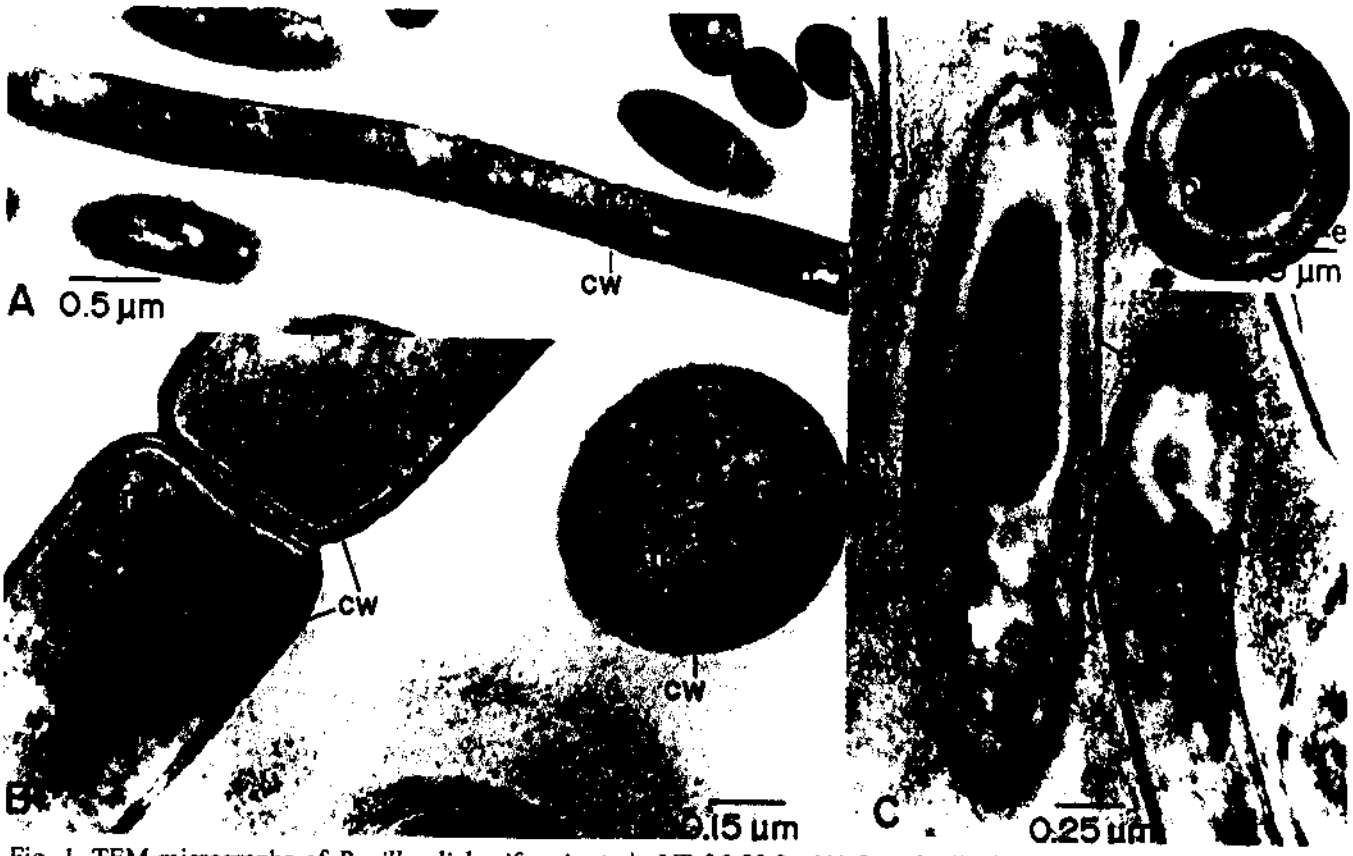

Fig. 1. TEM micrographs of *Bacillus licheniformis* strain NP-36-80-L. (A) Longitudinal section showing a filament of vegetative rod cells, surrounded by unilayer cell wall (cw). (B) High magnification showing the unilayer cell wall (cw) characteristic of the Gram-positive bacteria, and under the cytoplasmic membrane (m). (C) Sections that showing the endospore structure and the presence of a filamentous capsule or glicocalix (g). The inset show parts of the endospore protoplast (p), cortex (cx), and exosporium (e).

occurred singly or in chains. A capsule was detected by ink stain. In solid K medium and incubated at 30 °C, the vegetative cells develop endospores after 48 hours. The endospores were ellipsoidal, mainly in the subterminal position without swollen sporangium. In K broth medium, at 30 °C, polymorphic cells were developed. Transmission electron microscopy study obtained from *Bacillus licheniformis* strain NP-36-80-L, reveled Gram-positive organization of their vegetative cells by the presence of a thick, single-layer cell wall (Fig. 1A; 1B). This strain shows filaments cells, limited by cytoplasmic membrane surrounded by a fibrous capsule (or glicocalix), that was evident with ruthenium red (Fig. 1C). Chains of cells from *Bacillus licheniformis* strain NP-36-80-L exhibits a densely staining cytoplasm because of the presence of numerous ribosomes (Fig. 1A). Endospores were also studied by TEM. The ellipsoidal endospore, localized in subterminal position, was composed of a scarce cytoplasm bounded by a cortex and an exosporium (Fig 1C). The micrographs confirm the results of optical microscopy characterization.

We isolated the strains NP-36-80-G ("North Pond") and P2-V-83-2 ("Pentapus Salina"). They are phenotypically similar to NP-36-80-L. Colonies on modified K medium become opaque with a rough surface, and developed a filamentous shape, but they are easy to remove from the agar.

Microculture examination of the manganese-oxidizing bacteria isolates NP-36-80-L, NP-36-80-G, and P2-V-80-2 showed that vegetative-cell rods divided by binary fission, which resulted in chains that were quite long. They resembled a stellate structure attributed to *Metallogenium*, a mixed culture of fungi and bacteria.[16] Our *Metallogenium*-like bacterial colonies from the Baja California microbial mat are axenic culture phenomena. The hair-like growth and stellate shape was maintained in low to high magnification (Fig. 2). The paleobiological significance of *Metallogenium* phenomena, the supposed counterpart to *Eoastrion*, a 2-Ga-old microfossil, has been discussed in previous studies.[8, 16]

The strain P2-V-83-3 was isolated from "Pentapus Salina". This strain developed circular-shaped colonies, with erose margin, umbonate elevation, and easy to remove from the agar. After several days of incubation, the colonies become dark-brown in the center and light in the edge. The cell morphology corresponds to single and short chains of a motile, Gram-variable rod shape, 0.9-µm wide

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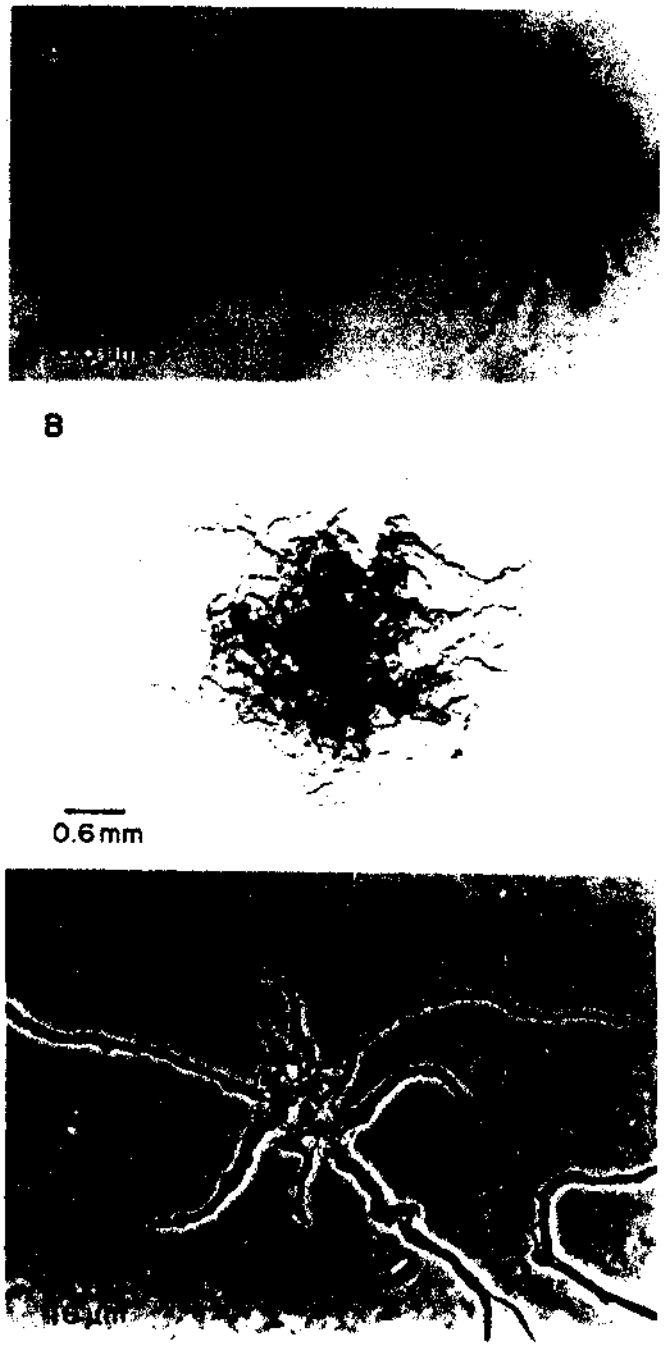

Fig. 2. *Eoastrion-Bacillus licheniformis*. (A) Two specimens of *Eoastrion* preserved in hematite, from a petrographic this section.[11] This preparation is from the Frere Formation, Western Australia ~ 1.7-Ga-old (Schopf 1983). (B) Colony filamentous shape growing on K medium of *B. licheniformis* NP-36-80-G. (C) Microcolony hair-like growth stellate structure conserved at higher magnification of strain NP-36-80-G.

and 3.5- to 5.0-µm long. After 48 h of incubation at 30 °C, rods were observed with ellipsoidal endospores in the central to terminal position, with swollen sporangium. In old cultures, clusters of spores were detected.

The bacterial identification shows that strains NP-36-80-L, NP-36-80-G, and P2-V-83-2 correspond to *Bacillus licheniformis* with a discrepancy in the position of the endospore, gelatin liquefaction, and use of citrate. The strain P2-V-83-3 was identified as *Bacillus brevis*, however showing differences in the cell dimensions and in casein hydrolysis (Table 1).

The strains isolated show different abilities to oxidize manganese (Table 2). The control medium without a manganese source show colonies without a brown color, and no blue color with LBB reagent. In contrast, the strains in K medium with manganese (Mn II) developed light to dark-brown colonies. The LBB spot test confirmed the presence of manganese oxide precipitates. For *Bacillus brevis* strain P2-V-83-3, the brown dark color of the colonies interfered in the LBB spot test. For this strain, the test was done under microscopic observation, in which the manganese oxide precipitates were associated with spore clusters from old cultures. *Bacillus licheniformis* strain NP-36-80-L from Baja California mats frequently lost the ability to oxidize manganese after several transfers.

DISCUSSION

This study describes four manganese-oxidizing bacterial strains isolated from microbial mats and identified at the species level by phenotypic characterization. Further studies on molecular taxonomy completed the identification of these new strains. *Bacillus licheniformis* strains show a distictive hair-like growth stellate structure, which has been considered the supposed counterpart to *Eoatrion,* a 2-Ga-old microfossil. The *B. licheniformis* strain NP-36-80-L developed variants without the ability to oxidize manganese. Meanwhile, *B. brevis* was more constant in the formation of manganese oxide precipitates. The presence of spores by these strains associated with the capability to precipitate manganese oxides, suggest an important role of this structure for explain ancient and modern MnO_2 deposits.

ACNOWLEDGMENTS

This study was supported in part by funds from the Facultad de Medicina, Universidad Nacional Autónoma de Mexico (UNAM), Project 177 (1980-1987). Scholarship from Programa de Superación del Personal Académico, UNAM (1986). I am grateful to Dr. Lynn Margulis for all the support received through the Planetary Biology and Microbial Ecology NASA Summer Research ProGram and Environmental Evolution ProGram, Boston University

Graduate School (1980-1986). The strain of *Bacillus subtilis* ATCC 6051e was obtained from Escuela Nacional de Ciencias Biológicas-IPN, Mexico, D.F. Thanks to Dr. Ellis Glazier, CIBNOR, for editing this English-language text.

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