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Isolation and Properties of a Denitrifying Bacterium Related to *Pseudomonas lemoignei*

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A previously undescribed denitrifying bacterium was isolated from soil. The cells are small, gram negative, slightly curved rods, asporogenous, and nonmotile. Motile clones, however, have been isolated from the nonmotile parent: these cells possess a single polar flagellum. The organism shows no fermenting activity and grows only in the presence of one of the following electron acceptors: NO_3^-, NO_2^-, N_2O, $S_4O_6^{2-}$, and O_2. It gives a positive oxidase test and has a cytochrome *c* and catalase. It requires no growth factors, is a chemoorganotroph, and uses only some alcohols and organic acids as carbon and energy supply. Poly-β-hydroxybutyrate is synthesized. The deoxyribonucleic acid base composition is 62.2 mol% guanine plus cytosine. The bacterium bears greatest resemblance to *Pseudomonas lemoignei*.

In a previous article we drew attention to the advantage of using nitrous oxide as a respiratory electron acceptor for the isolation of denitrifying bacteria by enrichment culture (7). In this manner, we have isolated an organism that is clearly different from the known denitrifiers of the genus *Pseudomonas*. This paper describes the bacterium.

MATERIALS AND METHODS

Isolation procedure. The mineral basal medium used for all media contained the following (per liter of distilled water): $Na_2HPO_4 \cdot 12H_2O$, 3.575 g; KH_2PO_4, 0.98 g; $MgSO_4 \cdot 7H_2O$, 0.03 g; NH_4Cl, 0.5 g; solution containing calcium and various heavy metals chelated with EDTA (ethylenediaminetetraacetic acid, disodium salt dihydrate, 50.0 g; $ZnSO_4 \cdot 7H_2O$, 2.2 g; $CaCl_2$, 5.54 g; $MnCl_2 \cdot 4H_2O$, 5.06 g; $FeSO_4 \cdot 7H_2O$, 4.79 g; $NH_4Mo_7O_{24} \cdot 4H_2O$, 1.1 g; $CuSO_4 \cdot 5H_2O$, 1.57 g; $CoCl_2 \cdot 6H_2O$, 1.6 g; H_3BO_3, 0.05 g; distilled water, 1,000 ml; adjusted to pH 6.0 with KOH), 0.2 ml; pH 7.0.

An enrichment culture was made in liquid medium containing 4.0 g of sodium succinate per liter under an atmosphere of pure N_2O. The inoculum was a sample of sulfide-containing mud from an anaerobic edge of a lagoon near the town of Berre (Etang de Berre), France. This body of water communicates with the sea. Primary cultures were kept at 32°C for a week and then serially transferred a number of times. Colonies of bacteria were isolated after transfer to solid medium (same medium containing 1.4% agar) and aerobic incubation at 32°C for 3 days. Clones were purified by several successive streakings and verified as pure when colonies of only one type were produced. The bacterium thus isolated can be maintained on nutrient agar but must be transferred every week.

Although several more enrichment cultures were made under the same conditions starting with various garden soil and sulfide-containing mud samples, we were not able to reisolate this bacterium.

Identification methods. The methods used in characterization were described previously (7, 9). Poly-β-hydroxybutyrate was identified by Sudan black staining and by extraction in hot $CHCl_3$. The extract was converted to crotonic acid by heating at 100°C in the presence of concentrated H_2SO_4 (4). Tetrathionate reduction was demonstrated by iodometric assay of the thiosulfate formed in anaerobic cultures.

Broth-grown cells (25°C) were absorbed on 300-mesh, Formvar-coated copper grids for 5 min at room temperature and stained with 2% sodium phosphotungstate at pH 7.5. Grids were examined in a Siemens electron microscope (Elmiskop IA) equipped with a 50-nm objective aperture and operated at 80 kV.

RESULTS

Morphology. Colonies are convex, translucent, circular with entire margins, slightly yellow, and do not exceed 3 mm in diameter on nutrient agar. They are neither coherent nor adherent to agar.

The short and slightly curved rods have rounded ends (1.2 to 2.4 μm by 0.6 to 0.8 μm) and multiply by binary fission. The original isolate was nonmotile, but motile clones have been isolated from the periphery of growth in semisolid media (4.0 g of agar per liter). These cells possess a single polar flagellum and are otherwise identical to the nonmotile parent. Cells are gram negative and form no endospores. Capsules were not observed. Examination of cells of the motile variant by electron microscopy confirms the presence of a polar flagellum and shows the absence of pili (Fig. 1). The clear zones probably

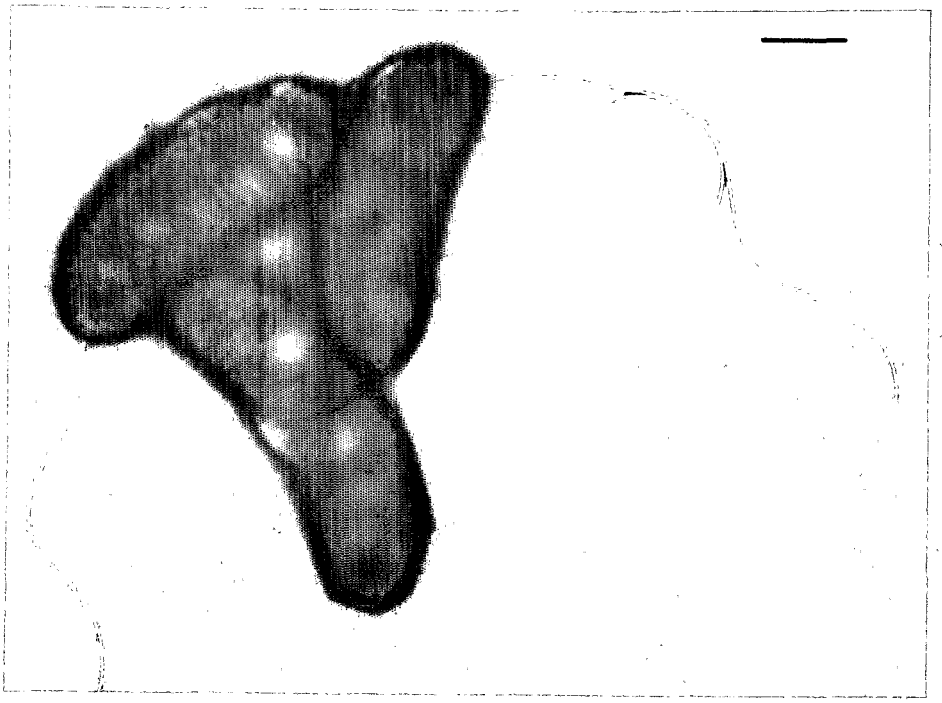

FIG. 1. *Electron micrograph of negatively stained cells of a motile clone. Bar represents 0.5 μm.*

represent deposits of poly-β-hydroxybutyrate because this compound is very electron transparent.

Cultural and physiological characters. The organism is a chemoorganotroph that does not grow in liquid mineral media under atmospheres containing H_2, O_2 (or N_2O), and CO_2, and does not require any growth factor. Metabolism is respiratory; using O_2, NO_3^-, NO_2^-, N_2O, or $S_4O_6^{2-}$ as substrates. Fermentative activity is completely lacking. The bacterium reduces nitrate to nitrite with feeble production of gas, but it denitrifies nitrous oxide vigorously. It grows in nutrient broth (Difco) at pH 9.0, but not in peptone water containing 3% NaCl. It does not grow at 4°C nor at 40°C. In nutrient broth (Difco), growth is flocculent and a pellicle is formed. No brown or black pigment is produced on nutrient agar (Difco) containing 10.0 g of tyrosine per liter.

The bacterium synthesizes poly-β-hydroxybutyrate when grown in medium containing DL-3-hydroxybutyrate. Purified poly-β-hydroxybutyrate (from *Bacillus megaterium*) is neither hydrolyzed nor assimilated. Metachromatic granules are not found within cells grown on nutrient agar.

The following compounds are utilized as sources of carbon and energy: ethanol, propanol, butanol, 1,2-propanediol, acetate, propionate, butyrate, isovalerate, succinate, fumarate, L-lactate, D-lactate, DL-3-hydroxybutyrate, L-malate, D-malate, pyruvate, α-ketoglutarate. The following compounds are not used as sources of carbon and energy: 2,3-butanediol, 1,2-ethanediol, glycerol, isobutanol, methanol, isobutyrate, valerate, caproate, pelargonate, caprate, malonate, glutarate, adipate, pimelate, suberate, sebacate, glycolate, citrate, DL-isocitrate, tartronate, β-hydroxy-β-methylglutarate, *d*-tartrate, *l*-tartrate, *meso*-tartrate, mucate, azelaidate, levulinate, maleate, itaconate, *trans*-aconitate, *cis*-aconitate, mesaconate, citraconate, crotonate, geraniol. None of the carbohydrates, amino acids, aromatic acids, nor amines tested (9) can serve as carbon and energy sources.

The organism is oxidase and catalase positive. It contains cytochrome *c* whose reduced form has major peaks of absorption at 551.5 nm (α) and at 522 nm (β). Neither pyocyanin nor fluorescent pigments are produced in the King A or King B media, respectively. Gelatin is not liquefied, and starch, Tween 80, and lecithin are not hydrolyzed. Urease is not produced, nor is indole. Hydrogenase is produced in anaerobiosis in a complex medium containing succinate and yeast extract under an atmosphere of H_2 and N_2O. L-Phenylalanine deaminase is not produced nor is constitutive arginine dihydrolase. Nitrogen is not fixed. Nitrate is assimilated. Ni-

trate reductase A is demonstrable in anaerobic culture with NO_3^-; nitrate reductase B is demonstrable in aerobic culture grown in the absence of NO_3^- (6). Extracts of cells cultivated anaerobically in the presence of NO_3^-, NO_2^-, or N_2O yield extremely little or no tetramethyl-*p*-phenylenediamine-nitrite reductase activity. Tetrathionate reductase is produced in anaerobiosis in a medium containing $S_4O_6^{2-}$.

Growth and denitrification of N_2O are obtained in complex media with yeast extract or in minimal media containing acetate, butyrate, succinate, DL-lactate, or L-malate. The dinitrogen produced in stoichiometric amounts from the reduction of N_2O in the presence of succinate by cell suspensions has been measured and identified by gas chromatography (3).

DNA base composition. The deoxyribonucleic acid (DNA) has a buoyant density in CsCl of 1.721 g/cm^3, indicating a base composition of 62.2 mol% guanine plus cytosine (G+C) (5, 8).

Taxonomy. The bacterium clearly is a member of the genus *Pseudomonas*. The species known to denitrify are *P. aeruginosa*, *P. fluorescens*, *P. stutzeri* (including *P. stanieri*), *P. mendocina*, *P. pseudomallei*, *P. mallei*, *P. solanacearum*, *P. caryophylli*, and *P. pickettii*. All of these are nutritionally omnivorous and assimilate a broad range of carbohydrates and amino acids (2, 9). Physiologically, the bacterium we have encountered and described bears greatest resemblance to *P. lemoignei*. The latter assimilates only acetate, butyrate, valerate, pyruvate, succinate, and DL-3-hydroxybutyrate, and accumulates poly-β-hydroxybutyrate as a reserve substance. It also is characterized by equally small colonies. However, *P. lemoignei* differs in growing at 41°C, in producing colonies that are very coherent and adherent to agar, in not assimilating DL-lactate, propionate, L-malate, and α-ketoglutarate, in failing to denitrify, and in being able to hydrolyze extracellular poly-β-hydroxybutyrate. Furthermore, its colonies and surrounding media turn brown or black when it is grown on complex media or media containing tyrosine and a suitable carbon source, and the G+C content of its DNA is 58.2 mol% (1).

DISCUSSION

We do not know whether the use of tetrathionate as a respiratory electron acceptor is specific to the bacterium described because the pseudomonads were never examined for this character.

Is this organism a new species or is it a denitrifying variety of *P. lemoignei*? Only by studying new strains can we determine its taxonomic position. Unfortunately there are serious difficulties in isolating such strains because the culture conditions used during enrichment are not selective. They allow the growth of other denitrifying bacteria such as *P. stutzeri* and *Alcaligenes denitrificans*. It should be remembered that only one strain of *P. lemoignei* has been isolated and described (1).

We have deposited the holotype in the collection of the Pasteur Institute (number CIP 301-75).

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REPRINT REQUESTS

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