The isolation and properties of a denitrifying bacterium of the genus *Flavobacterium*

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A previously undescribed denitrifying bacterium was isolated from soil. The cells are small gram-negative rods, asporogenous, and non-motile. Colonies become yellow after long exposure to light. This colouring is due to the production of a carotenoid pigment. The organism shows no fermenting activity, and grows only in the presence of one of the following electron acceptors: NO_2^-, N_2O, and O_2. It does not reduce nitrate. It gives a positive oxidase test and has a cytochrome *c* and catalase. It requires no growth factors, is a chemoorganotroph and uses only sugars as carbon and energy supply. The DNA base composition is 40.8 moles percent GC. Although presenting the physiological characteristics of a pseudomonad, the organism described has been placed in the genus *Flavobacterium* because of its pigmentation and its low GC percentage.

INTRODUCTION

Denitrifying bacteria are generally isolated from soil by enrichment in media containing nitrate. However, this compound is not used as an electron acceptor by *Alcaligenes odorans* var. *viridans* and by some strains of *Pseudomonas aeruginosa* that can, nevertheless, reduce nitrite. Moreover, nitrate reduction can entrain an accumulation of NO_2^- which is toxic. Nitrous oxide, which is an inter-

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mediate in the reduction of NO_3^- to N2, is also used as a respiratory substrate by these organisms (Pichinoty, 1973). This gas is very soluble in water and is non-toxic. By using enrichment cultures containing N_2O one should be able to isolate denitrifiers that are unable to reduce NO_3^-. To our knowledge bacteriologists have never taken advantage of these conditions of isolation.

MATERIALS AND METHODS

Organism. The mineral basal medium used for all media contained per liter 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; trace elements solution, 0.2 ml. The bacterium described in the present paper was isolated from garden soil by enrichment in a liquid medium containing 0.4% yeast extract (Difco) under an atmosphere of N_2O. Incubation was at 32 C. After three passages, colony isolation was effected on the same medium solidified with agar (1.4% w/v) and incubated aerobically at 32 C for 24 h. Small colonies were obtained which gave a prompt positive classical oxidase reaction (Skerman, 1967), as distinguished from the larger colonies of facultative anaerobes with fermentative ability which are not selected against by the media employed. Successive platings assured culture purity. Two flasks were then inoculated with a single isolate and one flushed with N_2O and the other evacuated. Only the former yielded abundant growth which was accompanied by the visible production of nitrogen gas bubbles. The bacterium which we have isolated can be maintained aerobically with no difficulty on nutrient agar.

Tests. The majority of the tests of phenotypic properties have been described by Stanier, Palleroni and Doudoroff (1966). Assimilation of various carbon sources was studied in liquid medium at 32 C. Growth was estimated visually after 7 days of incubation. The nitrate reductase activity of enzyme extracts was measured manometrically (Pichinoty and Piéchaud, 1968). Nitrite reductase was demonstrated by the production of NO with N,N,N′,N′-tetramethyl-*p*-phenylenediamine as electron donor (Miyata and Mori, 1968). Nitrogen formed in the course of anaerobic N_2O reduction in the presence of glucose was identified and quantified by gas chromatography (Garcia, 1974).

Electron micrographs were prepared of negatively-stained preparations (1% (w/v) ammonium molybdate or 1% (w/v) potassium phosphotungstate at pH 7.0).

Deoxyribonucleic acid was extracted from cells harvested from the complex medium as previously described (Mandel, 1966). The buoyant density in CsCl was determined (Mandel, Schildkraut and Marmur, 1968) and the base composition calculated by the method of Schildkraut, Marmur and Doty (1962).

Pigment was extracted from lyophilized cells which had been cultivated aerobically in the complex medium. Three successive extractions with methanol

at room temperature removed all visible colour (neither petroleum ether nor acetone accomplished any solubilization). Preparative thin-layer chromatography of the methanol extract was done on silicic acid plates (Merck) with successive development by CCl_4–hexane–ethyl acetate (5:1:3, v/v/v) followed by petroleum ether-acetone (9:1, v/v).

RESULTS

Morphology. Small rods with rounded ends or as coccobacilli; 0.5 μm width by 0.9–1.4 μm length. Division by binary fission. Motility has not been observed under any growth condition, gliding or twitching movements have not been observed though sought for; swarming at edges of colonies on nutrient agar is also lacking. Neither flagellae, pili, nor capsules were observed. Cells are gram-negative. Endospores are not formed. Colonies on agar are convex, smooth, glistening, opaque, circular with entire margins. Attaining 10 mm diameter, they appear cream-coloured by reflected light. Upon exposure to light a deep yellow colour appears after several days due to the production of a non-diffusible carotenoid.

Cultural and physiological characterization. The bacterium is a chemoorganotroph, not growing in mineral media in an atmosphere of H_2, O_2 (or N_2O) and CO_2. No growth factors are required. It is incapable of fermenting sugars. Oxygen, NO_2^- or N_2O are utilized as respiratory electron acceptors. Does not grow (or grows extremely slowly) anaerobically in the presence of nitrate ion. Rapid growth with dense and uniform turbidity is obtained in complex nutrient broths, both aerobically and anaerobically in the presence of N_2O. Filamentous and long cells are characteristic of growth on complex agar media. Does not grow either at 4 C or 40 C. Grows in broth at pH 9.0 and in peptone water with 3% NaCl, but not with 6% NaCl. The lipid inclusions of poly-β-hydroxybutyrate are not found in minimal glucose medium deficient in nitrogen. Metachromatic granules are found within cells grown on nutrient agar. Resistant to polymyxin B. Neither pyocyanin nor fluorescent pigments are produced in the King A or B media.

The following compounds are utilized under aerobic conditions as sources of carbon and energy: D-glucose, D-galactose, D-mannose, fructose, α-methyl-D-glucoside, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-mannoside, D-arabinose, L-arabinose, lactose, maltose, cellobiose, melibiose, trehalose, arbutin, D-melezitose, raffinose and ribitol. The following compounds are not utilized: L-glucose, L-sorbose, D-fucose, L-fucose, D-glucosamine, β-methyl-D-glucoside, salicin, D-gluconate, 2-keto-D-gluconate, D-glucuronate, D-saccharate, D-xylose, L-rhamnose, D-ribose, inosine, α-methyl-D-xyloside, β-methyl-D-xyloside, D-erythrose, saccharose, starch, inulin, D-mannitol, D-sorbitol, D-dulcitol, *meso*-erythritol, D-arabitol, L-arabitol, glycerol, glycerate,

ethylene glycol, propylene glycol and 2,3-butylene glycol. Primary alcohols, fatty acids, aliphatic dicarboxylic acids, hydroxyacids, ketoacids, amino acids, aromatic compounds and amines do not serve as sources of carbon and energy (Stanier et al., 1966).

The organism is oxidase- and catalase-positive. A cytochrome *c* with absorbance maxima at 552 nm (α) and 522.5 nm (β) in the reduced form is present. It does not hydrolyse gelatin, starch nor "Tween 80". Cellulose is not attacked. Acids are produced from glucose in the presence of air. Ketodisaccharides are not produced from lactose, sucrose, or maltose (Bernaerts and De Ley, 1963). Litmus milk is acidified with reduction of the dye. β-Galactosidase is present, urease is not. Very little (or no) indole is produced from tryptophan after several days of incubation. Lecithinase and hydrogenase are absent. Constitutive arginine dihydrolase is absent. L-Phenylalanine deaminase is produced. It does not fix N_2 nor assimilate nitrate and is nitrate reductase-negative. Respiratory nitrite reductase is present. It grows and denitrifies N_2O in a mineral medium containing any of the following carbohydrates: D-glucose, D-galactose, lactose, L-arabinose, D-arabinose, fructose or maltose.

The base composition of the DNA is 40.8 moles percent GC.

Identification of the pigment. The bright yellow pigment was extracted from cells with methanol. The pigment was resolved in four components by thin-layer chromatography (see Materials and Methods). The principal yellow pigment component migrated with an R_f of 0.20; three minor yellow components were resolved at R_f 0.30, 0.70 and 0.85. Under the same conditions, an authentic sample of lutein gave an R_f of 0.25. After spraying the plates with a solution of 50% sulfuric acid, a blue coloration which is characteristic of carotenoids, was observed at the sites of the four yellow pigments. The spectra of the principal carotenoid are shown in Fig. 1. They show the following absorbance peaks: in petroleum ether, 420, 445, and 475 nm; in benzene, 435, 460, and 488 nm; in carbon disulfide, (450), 478, and 510 nm. The principal pigment is therefore a xantophyll slightly more polar than lutein.

DISCUSSION

The GC content of the DNA of species of *Pseudomonas* range between 57 and 70 moles % (Mandel, 1966). The bacterium which we have described cannot be assigned to this genus. Nor can it be classed amongst the Enterobacteriaceae because of its cytochrome *c* oxidase activity and total lack of fermentative ability. Aerobic rods, gram-negative and not forming spores, some of which are pigmented yellow and have GC contents in the neighbourhood of 40% are presently found in the genera *Cytophaga* and *Flavobacterium*. The absence of "swarming", the immotility of the cells and the inability to degrade insoluble substrates such as cellulose preclude any assignment of this organism to the

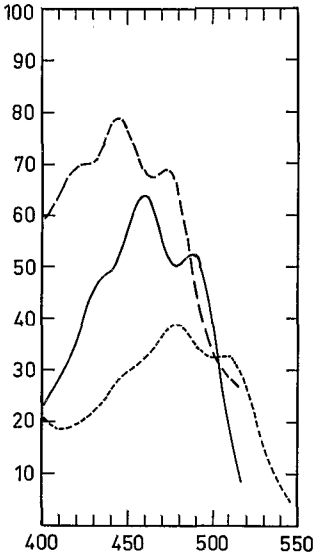

Fig. 1. Spectra of the principal carotenoid: ——, in benzene;
– – –, in petroleum ether;
- - - -, in CS_2.
Abscissa: wavelength (nm); ordinate: absorbance.

genus *Cytophaga* (or to other genera of the Flexibacterales). It corresponds therefore, most closely to those low-GC organisms currently grouped in *Flavobacterium* (Weeks, 1974). Species assigned to this genus have been poorly studied and their published descriptions do not contain more than a very limited number of biochemical characters. Further, their abilities to denitrify nitrite and nitrous oxide have never been tested.

The bacterium which we have described exhibits one original feature; it is the sole denitrifier known which is restricted to using carbohydrates.

The culture has been deposited as N°. CIP 12–75 in the Collection of the Pasteur Institute.

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