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Degradation of hydroxyhydroquinone by the strictly anaerobic fermenting bacterium *Pelobacter massiliensis* sp. nov.

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Abstract. A new rod-shaped, gram-negative, non-spore-forming, strictly anaerobic bacterium (strain HHQ7) was enriched and isolated from marine mud samples with hydroxyhydroquinone (1,2,4-trihydroxybenzene) as sole substrate. Strain HHQ7 fermented hydroxyhydroquinone, pyrogallol (1,2,3-trihydroxybenzene), phloroglucinol (1,3,5-trihydroxybenzene) and gallic acid (3,4,5-trihydroxybenzoate) to 3 mol acetate (plus 1 mol CO_2 in the case of gallic acid) per mol of substrate. Resorcinol accumulated intermediately during growth on hydroxyhydroquinone. No other aliphatic or aromatic substrates were utilized. Sulfate, sulfite, sulfur, nitrate, and fumarate were not reduced with hydroxyhydroquinone as electron donor. The strain grew in sulfide-reduced mineral medium supplemented with 7 vitamins. The DNA base ratio was 59% G + C. Strain HHQ7 is classified as a new species of the genus *Pelobacter, P. massiliensis*. Experiments with dense cell suspensions of hydroxyhydroquinone- and pyrogallol-grown cells showed different kinetics of hydroxyhydroquinone and pyrogallol degradation, as well as different patterns of resorcinol accumulation, indicating that these substrates are metabolized by different transhydroxylation reactions.

Key words: Hydroxyhydroquinone – Anaerobic degradation – *Pelobacter massiliensis* sp. nov. – Trihydroxybenzenes – Aromatic compounds

The three trihydroxybenzene isomers occur with different abundancies in nature. Pyrogallol and its derivatives are major products of lignin degradation (Young and Frazer 1987), occur in tannins of gall nuts and lichens, are intermediates in the synthesis of phytohormones, and are secondary metabolites of many plants and fruits (Beyer and Walter 1988). Phloroglucinol is a structural component of flavonoid compounds and anthocyanins, and an intermediate in anaerobic pyrogallol degradation by *Pelobacter acidigallici* (Samain et al. 1986) and *Eubacterium oxidoreducens* (Krumholz et al. 1987). Free hydroxyhydroquinone occures in the marine sponge *Axinella polycapella*; its presence there is attributed to its antibacterial and antifungal effect (Cimino et al. 1974; Wratten and Meinwald 1981). Intermediate accumulation of hydroxyhydroquinone was reported for aerobic degradation of phloroglucinol by *Fusarium solani* (Walker and Taylor 1983) and by *Penicillium simplicissimum* (Patel et al. 1990), and for anaerobic phloroglucinol breakdown by *Rhodocyclus gelatinosa* (formerly *Rhodopseudomonas gelatinosa*, Evans 1977). Obligately anaerobic bacteria utilizing hydroxyhydroquinone are so far unknown. In this study we describe the enrichment and isolation of strictly anaerobic bacteria with hydroxyhydroquinone as sole carbon and energy source.

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Material and methods

Sources of organisms

Strain HHQ7 was isolated from an enrichment culture with hydroxyhydroquinone inoculated with black sediment from Etang de Prevost, a salt marsh near Marseille, France. Other enrichments were performed with anorganic-rich black sediment from Rio de la Pergola, a canal in Venice, Italy.

Media and growth conditions

Anaerobic, defined mineral media of various salinities were prepared as described by Widdel and Pfennig (1984), using trace element solution SL10a (Widdel 1986), 2 ml per l medium. Selenite-tungstate solution was added to a final concentration of 20 nM each. Six-vitamin solution, vitamin B_{12} and substrates were filter-sterilized and added to the autoclaved medium before inoculation. Utilization of substrates was tested in 20 ml screw cap tubes, following growth by turbidity measurements at 450 nm in a Spectronic 20 photometer (Milton Roy, Rochester, NY, USA). All growth tests were carried out at least in duplicate at 30° C in the dark unless otherwise indicated. Aerobic growth was tested in tubes containing complex me-

dium with 1% agar. Fermentation stoichiometries were determined using 0.5 l infusion bottles sealed with rubber septa and gassed with N_2/CO_2 (90/10, v/v). Optical density and the concentrations of hydroxyhydroquinone, acetate, and resorcinol were measured at regular time intervals. Growth yield was determined in 1 l batch cultures (Widdel and Pfennig 1981).

Isolation and purity control

Pure cultures were obtained by repeated application of agar dilution series as described by Widdel and Pfennig (1984). Purity was checked microscopically and by testing aerobic and anaerobic growth in complex media (AC-medium, Difco Laboratories, Detroit, Mich., USA, and in a medium containing 0.1% yeast extract, 5 mM fumarate, 5 mM pyruvate, 3 mM glucose with or without 4 mM hydroxyhydroquinone added).

Gram staining was performed according to Magee et al. (1975) with *Acetobacterium woodii* NZva16 and *Escherichia coli* K12 as references.

Pigment analysis

For detection of cytochromes, redox difference spectra of cellfree extracts were recorded using an Uvicon 860 spectrophotometer (Kontron, Zürich, Switzerland).

Experiments with dense cell suspensions

Cells were grown in 100 ml infusion bottles under N_2/CO_2 and harvested in the late exponential growth phase by centrifugation in the culture bottles at $3000 \times g$ for 20 min. Cells were washed once and resuspended to a final density of 625 μg protein · mg^{-1} in 50 mM N_2-sparged sodium phosphate buffer, pH 7.0, containing the same amounts of NaCl and $MgCl_2$ as the medium to prevent lysis. Assays were performed in N_2-gassed vials with butyl rubber septa at room temperature. The reaction mixture consisted of cell suspension (250 μg protein), substrate, and N_2-gassed sodium phosphate buffer, in a final volume of 2 ml. All additions and samples were handled with gas-tight Unimetrics microliter syringes to prevent access of air. Samples (100 μl) were taken at regular intervals, injected into 400 μl of 0.1 M H_3PO_4, and kept on ice to be analyzed within 1 h.

Chemical analyses

Aromatic compounds were analyzed with a high-pressure liquid chromatograph equipped with a reverse phase (C_{18}) column (Brune and Schink 1990). A mobile phase consisting of 100 mM ammonium phosphate buffer, pH 2.6, and varying amounts of methanol were used. Acetate and other volatile fatty acids were assayed by gas chromatography (Dehning et al. 1989). Sulfide formation was analyzed according to Cord-Ruwisch (1985). Formation of nitrite from nitrate was assayed following azo dye formation with sulfanilic acid and α-naphthylamine (Procházková 1959). Protein was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Results

Enrichments and isolation of pure cultures

Enrichment cultures with sediment samples from Etang de Prevost in saltwater medium containing 10 mM sulfate and 1 mM hydroxyhydroquinone showed sulfide production after 3 months. Repeated addition of 1 mM hydroxyhydroquinone increased the sulfide concentration. After four transfers, flocs of bacteria were observed, consisting mainly of thin rods and short thick rods. The suspension was homogenized and agar dilution series were prepared with 2 mM hydroxyhydroquinone and 10 mM sulfate. Yellowish-brown satellite colonies developed around yellowish-white central colonies. The former consisted of short thick rods and could not be isolated with hydroxyhydroquinone and sulfate. However, they grew well with acetate and sulfate, and strongly resembled the typical acetate-degrading sulfate reducers of the genus *Desulfobacter*. The thin rods of the central colonies were isolated from subsequent agar dilution series with 2 mM hydroxyhydroquinone and 10 mM sulfate, but were shown not to form sulfide from sulfate during growth on hydroxyhydroquinone. Strain HHQ7 was maintained in pure culture for further studies.

Other enrichments with 1 mM hydroxyhydroquinone and 10 mM sulfate, inoculated with mud from Venice, produced sulfide and yielded morphologically similar bacteria as described above.

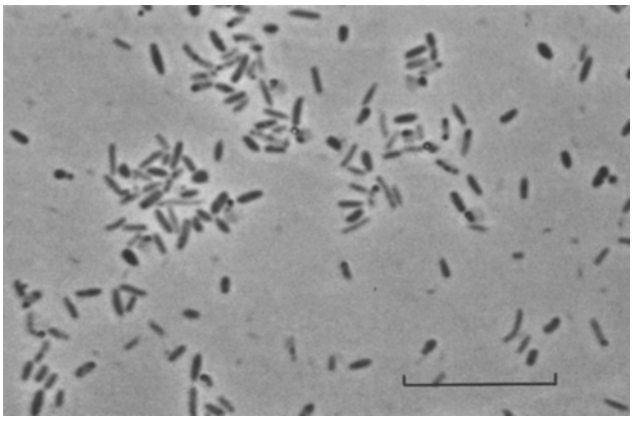

Fig. 1. Phase-contrast photomicrograph of strain HHQ7. *Bar*: 10 μm

Morphology

Figure 1 shows a photomicrograph of strain HHQ7 grown with hydroxyhydroquinone. The thin rods ($0.4-0.5\ \mu m \times 0.7-1.5\ \mu m$) were frequently irregular in length, width, and refractility when grown on this substrate. On pyrogallol, phloroglucinol, and gallate, the rods were more regular and thicker. Motility was observed occasionally in young cultures. The cells tended to grow on the glass surface of the culture bottles. Strain HHQ7 stained gram-negative; endospores were never observed.

Physiological and nutritional properties

Strain HHQ7 grew in saltwater medium as well as in brackish medium. No growth occurred in freshwater me-

Table 1. Compounds tested as possible growth substrates for strain HHQ7. Substrate concentrations in mM are given in parentheses

Substrates supporting growth:

Hydroxyhydroquinone (4), pyrogallol (4), phloroglucinol (4), gallic acid (4).

Substrates not supporting growth:

Phenol (1), catechol (1), resorcinol (2), hydroquinone (2), *o*-cresol (1), *m*-cresol (1), *p*-cresol (1), benzoate (2), 2-hydroxybenzoate (2), 3-hydroxybenzoate (2), 4-hydroxybenzoate (2), 2,3-dihydroxybenzoate (2), 2,4-dihydroxybenzoate (2), 2,5-dihydroxybenzoate (2), 3,5-dihydroxybenzoate (2), 2,6-dihydroxybenzoate (2), 2,3,4-trihydroxybenzoate (2), phenylacetate (2), 3,4,5-trimethoxycinnamate (2), vanillate (2), quinate (2), cyclohexanecarboxylate (2), *m*-inositol (2), cyclohexanol (2), cyclohexanone (2), phenylalanine (2), tyrosine (2), tryptophan (2), aniline (1), 2-aminobenzoate (2), 3-aminobenzoate (2), 4-aminobenzoate (2), methanol (5), ethanol (5), lactate (5), pyruvate (5), 3-hydroxybutyrate (5), malonate (5), succinate (5), fumarate (5), glutarate (5), malate (5), L-tartrate (5), citrate (5), glycollate (5), glyoxylate (5), crotonate (5), ethyleneglycol (5), glycerol (5), glucose (10), fructose (10), mannose (10), xylose (10)

dium or in saltwater medium supplemented with additional NaCl (400 mM) and $MgCl_2$ (25 mM). No growth was found in complex medium under aerobic or microaerobic conditions. Addition of yeast extract, pig manure filtrate, bovine rumen fluid or a mixture of fatty acids (Widdel 1980) did not enhance growth rate or growth yield. Hydroxyhydroquinone, pyrogallol, phloroglucinol, and gallic acid were the only substrates utilized by strain HHQ7. Hydroxyhydroquinone or pyrogallol at concentrations above 4 mM retarded growth significantly. The maximum substrate concentration tolerated was 8 mM hydroxyhydroquinone or pyrogallol, causing a lag phase of 3 days. No other substrates, including other aromatic compounds, mono- and dicarboxylic acids, alcohols and sugars, were utilized (Table 1). Neither nitrate (5 mM), sulfate (10 mM), sulfite (3 mM), thiosulfate (6 mM), sulfur, nor fumarate (10 mM) was used as electron acceptor during degradation of hydroxyhydroquinone. No growth occurred below 20° C or above 40° C. Growth was fastest at 30° C; the respective doubling times were 50 h with hydroxyhydroquinone and 36 h with pyrogallol as substrate. The pH range of growth was pH 6.5 to 7.8, with an optimum at pH 7.2.

Stoichiometries and growth yields

Strain HHQ7 degraded hydroxyhydroquinone, pyrogallol, phloroglucinol and gallic acid to 3 mol acetic acid per mol of substrate (Table 2), according to the following equations:

$$C_6H_6O_3 + 3\,H_2O \rightarrow 3\,CH_3COO^- + 3\,H^+$$

$$C_7H_5O_5^- + 4\,H_2O \rightarrow 3\,CH_3COO^- + HCO_3^- + 3\,H^+.$$

Small amounts of resorcinol, in addition to acetate, were produced only during growth on hydroxyhydroquinone, but not on the other substrates. Figure 2 represents the typical time course of hydroxyhydroquinone fermentation by strain HHQ7, showing a transient accumulation of resorcinol. For growth on hydroxyhydroquinone, a molar growth yield (Y_s) of 3.9 g/mol hydroxyhydroquinone was determined. Y_s for the other substrates were calculated using the OD/dry cell matter correlation determined on hydroxyhydroquinone. The respective values are 5.5, 7.4, and 8.6 $g \cdot mol^{-1}$ for phloroglucinol, pyrogallol, and gallic acid.

Pigments and DNA base composition

Redox difference spectra of dithionite-reduced minus air-oxidized cell extracts showed no pigments absorbing in the range of 350–650 nm (with a protein content of about 0.5 mg/ml), thus providing no indication for the

Table 2. Fermentation balances for growth of strain HHQ7 on trihydroxybenzenes and gallic acid

Substrate	Amount of substrate consumed (mmol)	Acetate produced (mmol)	Resorcinol produced (mmol)	Dry cell matter formed[a] (mg)	Substrate assimilated[b] (mmol)	Substrate dissimilated (mmol)	Carbon recovery (%)
Hydroxyhydroquinone	0.97	2.74	0.023	2.7	0.018	0.929	98
	1.84	5.40	0.045	7.4	0.051	1.744	103
	2.90	8.45	0.063	11.7	0.080	2.757	102
	3.95	10.83	0.083	15.2	0.104	3.763	96
Phloroglucinol	3.16	8.59	–	17.3	0.119	3.040	94
Pyrogallol	3.92	10.75	–	28.9	0.199	3.721	96
Gallic acid	3.82	10.26	–	32.8	0.225	3.595	95

[a] Calculations are based on a correlation of OD with dry cell matter determined during growth on hydroxyhydroquinone

[b] Substrate consumed for cell matter synthesis was calculated by the following equations:

$$17\,C_6H_6O_3 + 39\,H_2O \rightarrow 24\,(C_4H_7O_3) + 6\,HCO_3^- + 6\,H^+$$
$$17\,C_7H_6O_5 + 56\,H_2O \rightarrow 24\,(C_4H_7O_3) + 23\,HCO_3^- + 23\,H^+$$

6.87 mmol trihydroxybenzenes or gallic acid were assimilated into 1 g of dry cell matter

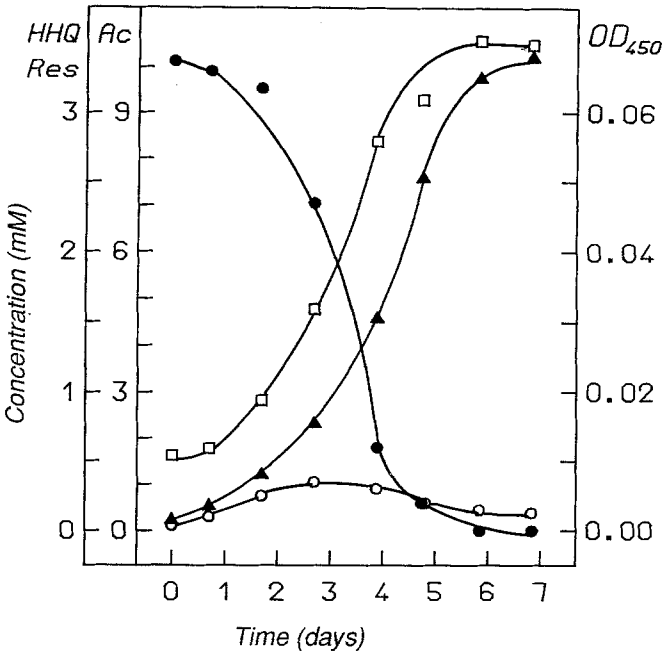

Fig. 2. Time course of hydroxyhydroquinone fermentation by strain HHQ7. (●) Hydroxyhydroquinone (HHQ), (□) optical density, (▲) acetate (Ac), and (○) resorcinol (Res)

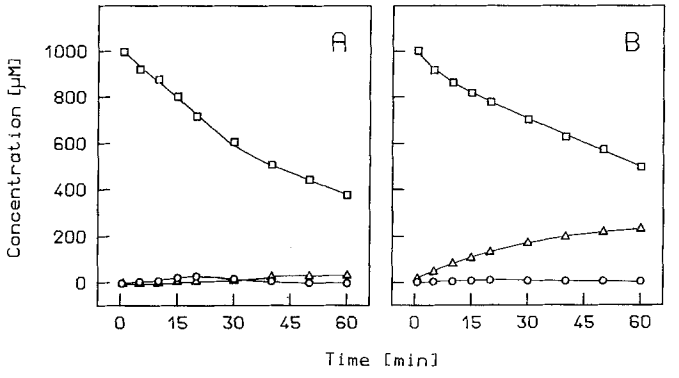

Fig. 3. Degradation of hydroxyhydroquinone (□) by dense cell suspensions of strain HHQ7, grown on hydroxyhydroquinone (A) or pyrogallol (B). (○) Phloroglucinol, (△) resorcinol. Protein concentration in both assays was 125 µg · ml^{-1}

presence of cytochromes. The G + C content of the DNA was 59 ± 2 mol% as determined by the thermal denaturation method (Marmur and Doty 1962).

Experiments with dense cell suspensions

Substrate degradation was studied in dense cell suspensions. While cells grown on hydroxyhydroquinone degraded hydroxyhydroquinone without significant accumulation of aromatic metabolites, pyrogallol-grown cells converted hydroxyhydroquinone to resorcinol at a 2:1 ratio (Fig. 3). Another significant difference between hydroxyhydroquinone- and pyrogallol-grown cells became evident during pyrogallol degradation (Fig. 4): Hydroxyhydroquinone-grown cells degraded pyrogallol immediately, while pyrogallol degradation by pyrogallol-grown cells was reproducibly biphasic. After an initial lag phase that varied in length with different cell extracts, the rate of pyrogallol consumption increased approximately threefold. Small amounts of phloroglucinol accumulated under both conditions.

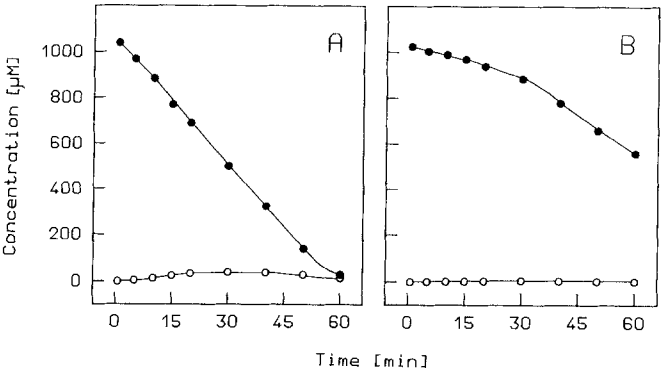

Fig. 4. Degradation of pyrogallol (●) by dense cell suspensions of strain HHQ7, grown on hydroxyhydroquinone (A) or pyrogallol (B). (○) Phloroglucinol. Protein concentration in both assays was 125 µg · ml^{-1}

Discussion

Physiology

Strain HHQ7 is the first obligately anaerobic bacterium known to grow with hydroxyhydroquinone as sole energy and carbon source. It degrades hydroxyhydroquinone fermentatively to 3 acetate; small amounts of resorcinol are produced. At this stage of the investigation we cannot explain why the resorcinol formed as an intermediate or by-product of the hydroxyhydroquinone degradation is at least partially reutilized by growing cultures, while resorcinol alone is not metabolized by strain HHQ7. The only other substrates used by strain HHQ7 are pyrogallol, phloroglucinol, and gallic acid. In this respect, strain HHQ7 strongly resembles *P. acidigallici*, but differs in its additional ability to degrade the third isomer of trihydroxybenzene, hydroxyhydroquinone.

These findings prove the existence of a new degradative capacity in anaerobic bacteria; the question arises how hydroxyhydroquinone is shuttled into the catabolic sequences of trihydroxybenzene fermentations.

Earlier investigations on the transformation of pyrogallol to phloroglucinol by *P. acidigallici* described a novel transhydroxylation reaction transferring a hydroxyl group from a hydroxyl donor (1,2,3,5-tetrahydroxybenzene) to pyrogallol, thus forming the product phloroglucinol and regenerating the cosubstrate (Brune and Schink 1990). The enzymatic activity in cell extracts of this bacterium proved to be relatively unspecific; several di- and trihydroxybenzenes could replace the physiological hydroxyl donor or acceptor molecule, but transhy-

droxylation of hydroxyhydroquinone to phloroglucinol was never observed.

The results of our incubation experiments indicate that also strain HHQ7 degrades pyrogallol through phloroglucinol as intermediate, and that also hydroxyhydroquinone is degraded via phloroglucinol. However, two different enzyme systems appear to be responsible for transformation of either substrate: While hydroxyhydroquinone-grown cells are adapted to degradation of both substrates without significant accumulation of intermediates, pyrogallol-grown cells accumulate large amounts of resorcinol when incubated with hydroxyhydroquinone. Apparently, pyrogallol-grown cells of strain HHQ7 possess the same transhydroxylating activity as already observed with *P. acidigallici* (Brune and Schink 1990). However, cell extracts of this bacterium did not transform hydroxyhydroquinone to phloroglucinol, neither in the presence nor in the absence of the tetrahydroxybenzene cosubstrate. Obviously hydroxyhydroquinone-grown cells of strain HHQ7 employ a different type of transhydroxylation reaction, probably involving a different tetrahydroxybenzene intermediate.

We are presently studying the transhydroxylation reactions catalyzed by cell extracts of strain HHQ7, as well as the further pathway of phloroglucinol degradation.

Strain HHQ7 is highly specialized with respect to its range of degradable substrates. The abundance of hydroxyhydroquinone in anaerobic sediments has not been investigated so far, but may play a more important role there than in aerobic environments since oxygen causes a rapid polymerization of the monomers. Gallic acid and pyrogallol are released in lignin degradation and may serve as natural substrates as well.

Taxonomy

The morphological, physiological and biochemical characteristics of the hydroxyhydroquinone-fermenting strain HHQ7 do not allow an unequivocal classification among known species. The DNA base ratio of 59 mol% G + C and the morphology of the rod-shaped, gram-negative bacteria allow the affiliation of strain HHQ7 with the genus *Pelobacter*. Most physiological properties of strain HHQ7 are similar to those of *Pelobacter acidigallici*; both degrade gallic acid, pyrogallol and phloroglucinol. However, strain HHQ7 possesses the unique ability to ferment hydroxyhydroquinone to acetate. It differs from *P. acidigallici* also with respect to cell size and the G + C content of the DNA. In order to appropriately classify strain HHQ7, we propose to establish it as a new species, *Pelobacter massiliensis*.

Pelobacter massiliensis

mas.si.li.en'sis. M. L. adj. massiliensis, a citizen of Massilia (lat.), Marseille, *Pelobacter massiliensis*, a strictly fermenting bacterium enriched from the sediments of Marseille.

Rod-shaped cells, 0.4 – 0.5 µm wide and 0.7 – 1.5 µm long. Gram-negative, non-spore-forming, motile in young cultures. Strictly anaerobic chemoorganotroph. Hydroxyhydroquinone, pyrogallol, phloroglucinol and gallic acid are the only fermentable substrates, and acetate, CO_2 and traces of resorcinol are the only fermentation products. Sulfate, sulfite, thiosulfate, sulfur, nitrate or fumarate are not reduced. Growth requires mineral medium with at least 10 g NaCl and 1.5 g $MgCl_2 \cdot 6\ H_2O$ per l, and sulfide as reducing agent. pH-optimum at pH 7.2, temperature optimum at 30° C. Cytochromes not detectable. DNA base ratio 59% G + C (thermal denaturation).

P. massiliensis was enriched and isolated so far only from marine sediments. Type strain: HHQ7, DSM 6233, deposited in: Deutsche Sammlung von Mirkoorganismen und Zellkulturen GmbH, Braunschweig, FRG.

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