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SHORT COMMUNICATION

Utilization of hexamethylenetetramine (urotropine) by bacteria and yeasts

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Abstract A slow growing bacterial population able to utilize hexamethylelenetetramine (urotropine) as sole source of carbon, nitrogen and energy was isolated from soil. From this crude enrichment culture two bacteria were isolated and identified as *Brevundimonas diminuta* and a *Phyllobacterium* sp. by sequencing of 16S ribosomal DNA. These bacteria also grew on urotropine but at a lower rate than the enrichment culture. Addition of glucose to the latter resulted in growth of some yeasts that overgrew the bacteria. Assimilation of urotropine as sole nitrogen source is very common among yeasts, 46 out of 60 species tested showed this characteristic.

Keywords *Brevundimonas diminuta* · Hexamethylenetetramine · *Phyllobacterium* sp. · Urotropine · Yeasts

Hexamethylenetetramine, systematic name 1,3,5,7-tetraazotricyclo-[3.3.1.1^{3,7}]-decane, is a colourless solid compound that is spontaneously formed in an aqueous solution of ammonia and formaldehyde. It has a cage-like structure and is composed of 6 mol of formaldehyde and 4 mol of ammonia. Hexamethylenetetramine is chemically stable, has a melting point of 280°C, but is subject to slow hydrolysis at pH lower than 5.0. Since the nineteenth century the compound has been applied to cure bladder infections. From this practice the trivial name ‘‘urotropine’’ originated. For reason of conciseness the latter name will be used in this paper. Lingvall and Lättemäe (1999) used the name hexamine. They added the compound to grass silage in order to improve hygienic quality and storage stability.

Little is known about the metabolic fate of urotropine in nature. Its building block formaldehyde is an intermediate of methanol catabolism. It is also formed by oxidation of methyl ethers and methylamines. Kaszycki and Koloczek (2002) reported degradation of urotropine by activated sludge augmented with the methylotrophic yeast *Hansenula polymorpha*, at pH 5.5 or lower. Whether urotropine degradation in this system is an enzymatic process or the result of spontaneous hydrolysis is not clear from this paper. In the present study attempts were made to isolate microorganisms from soil able to utilize urotropine as sole carbon or nitrogen source.

A solution of 10 mmol (1.4 g) of urotropine in 20 ml 1 M potassium phosphate buffer pH 6.0 was thoroughly mixed through 1 kg of soil. Two soils from grass-covered grounds were used. One was a sandy soil from a lawn (pH 6.1; water content 18%), the other was composed of sand

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and clay (pH 6.5; water content 30%). The soil samples were incubated at 20°C and were mixed daily. After 2 weeks about 10 g was used as inoculum in 200 ml mineral growth medium GB pH 6.1 (Middelhoven et al. 1991) with 10 mM urotropine (1.4 g/l) as sole carbon and nitrogen source, sterilized by heating in a microwave oven to the boiling point. GB medium has the composition of Difco Yeast Nitrogen Base but the concentration of phosphate buffer was tenfold higher and ammonium sulphate was omitted. The enrichment culture was incubated at 20°C in a 500 ml erlenmeyer flask on a rotary shaker at a speed of 100 rpm. After one week, 1 ml was transferred to 50 ml similar medium, but with 20 mM urotropine, in a 200 ml erlenmeyer flask. After 2 weeks no growth was discernible. The cultures were divided in two equal parts, each incubated in a 100 ml erlenmeyer flask. To one of these, 1 ml of 50% sterile glucose solution was added; the other served as a control.

The enrichment culture without glucose, inoculated with the clay–sand soil, showed growth after 2 months. Some yeasts and many small rods were present. Pure cultures of bacteria were obtained by streaking on nutrient broth agar plates (per litre: 10 g peptone and 5 g yeast extract). These failed to grow in GB urotropine broth. The enrichment culture was inoculated into fresh 20 mM urotropine medium, 0.2 ml in 20 ml. Again growth occurred after 2 months. This could be repeated several times over a period of one and a half year. Eventually, a bacterial population was selected that showed growth in about 3 weeks at 20°C and at 30°C. Yeasts had vanished and small rods predominated.

In an attempt to increase the growth rate, some formaldehyde-generating C1 compounds were added to the enrichment culture at 30°C. Methanol, methylammonium chloride and sodium formate had no effect. Formaldehyde and paraformaldehyde were inhibitory. From the enrichment culture at 30°C a bacterial culture, Uro-30, could be isolated on nutrient broth agar that was able to grow in urotropine broth, albeit slowly, 30 days of incubation being required. Dr. Elke Lang of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) identified strain Uro-30 as *Brevundimonas diminuta*. A sequence of 674 base pairs of the 16S rRNA gene (GenBank accession number DQ768098) showed 100% identity with that of the type strain of *B. diminuta* (Segers et al. 1994).

In order to check whether this culture is representative for the population, a denaturing gradient gel electrophoresis (DGGE), according to van Doesburg et al. (2005) was carried out for the crude enrichment culture and for culture Uro-30 (Fig. 1). It can be seen that the amplicon derived from strain Uro-30 is not dominantly present in the enrichment culture, suggesting that this bacterium takes only a minor part in the crude culture.

Amplification of the 16S rDNA genes, cloning and sequencing (van Doesburg et al. 2005) was done for the crude culture. Total 16S rRNA gene sequences were compared to sequences deposited in publicly accessible databases using NCBI Blast search tool at http://www.ncbi/nlm.nih.gov/blast. The 16S rDNA gene sequence belonging to the most dominant amplicon in the enrichment culture showed >99% homology with two species of *Phyllobacterium* (6 and 7 base pairs different of

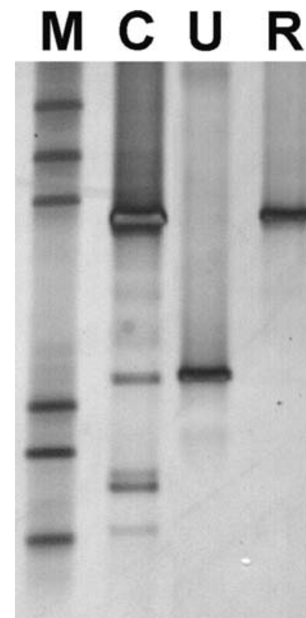

Fig. 1 DGGE analysis of amplified 16S rRNA genes (V6–V8 region) derived from the crude enrichment culture (C), *Brevundimonas diminuta* strain Uro-30 (U) and *Phyllobacterium* sp. strain URh-5 (R). Lane M is a DGGE marker

1447 tested). One of these strains had been identified as *Phyllobacterium trifolii* (Valverde et al. 2005, GenBank accession number AY786080); the other one is an uncultured *Phyllobacterium* strain (Barbieri et al. 2005, GenBank Accession Number AY599681). Attempts were made to isolate these by streaking on a selective medium, i.e. *Rhizobium* agar (per litre demineralized water: 10 g D-mannitol, 1 g yeast extract, 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 3 g $CaCO_3$, 15 g agar, sterilized at 120°C) and were checked for growth in urotropine broth. Strain URh-5 initially showed this character that however was lost during subcultivation on *Rhizobium* agar. Strain URh-5 showed only scant growth on nutrient agar. Both bacterial strains were deposited in the DSMZ culture collection (Braunschweig, Germany). *B. diminuta* Uro-30 received accession number DSMZ 17668, that of *Phyllobacterium* sp. URh-5 is DSM 17640. The 16S rDNA sequence of *Phyllobacterium* sp. URh-5 was deposited at GenBank, accession number DQ 431466. The crude enrichment culture is conserved in lyophilized state in the DSMZ culture collection, accession number DSM 17745.

After a few days the enrichment culture supplied with glucose showed abundant growth of yeasts. Pure cultures were obtained by streaking on glucose (1%), peptone (0.5%) yeast extract (0.3%) malt extract (0.3%) agar (2%). Cultures of ascomycetous yeasts were maintained on slants of the same medium, basidiomycetous yeasts on potato dextrose agar (Difco). The strains were examined for morphological and physiological properties with standard yeast identification methods (Yarrow 1998; Barnett et al. 2000). Utilization of carbon (10 g/l) and nitrogen sources (40 mM assimilable N) in liquid Difco Yeast Nitrogen Base and Yeast Carbon Base (2 ml in culture tubes of 18 mm diameter) was tested at 25°C on a rotary shaker at a speed of 100 rpm. Utilization of nitrite was tested by the auxanographic technique. The pH of growth media was adjusted to 5.5 if required, but the pH of media with galacturonic or quinic acid was not adjusted, which is in agreement with the laboratory practice of CBS Utrecht (D. Yarrow, personal communication; cf. Middelhoven, 1997). Growth on L-malic, D-galactaric (mucic, 2.5 g per litre), D-glucaric (saccharic) and tartaric acids was tested at pH 4.0.

Both soils yielded different yeast populations. The sandy clay soil was inhabited by *Trichosporon laibachii* (Windisch)Guého et M.Th. Smith and by a methylotrophic yeast identified as *Pichia methanolica* Makiguchi (Kato et al. 1974). The anamorphic basidiomycete *T. laibachii* UK-201 was identified by the keys provided by Middelhoven (2004) and Middelhoven et al. (2004), based on growth tests on benzene compounds, polysaccharides, uric acid and other non-conventional carbon sources. *P. methanolica* UK-202 (CBS 9489) was identified by the keys provided by Barnett et al. (2000) and Payne et al. (1998). The strain differed from the other ones known in the literature by failing to assimilate galactose and galactitol. CBS 9489 differed from the type strain of *P. methanolica* (CBS 6515) by six base pairs in the D1D2 region of 26S rRNA gene (V. Robert, personal communication). This would justify introduction of a novel species if more physiological characters would be different. *P. methanolica* is a soil inhabitant, isolated from soils in Japan and The Netherlands.

The sandy soil from a lawn yielded one yeast type that took an intermediate position between *Leucosporidium scottii* Fell, Statzell, I.L. Hunter and Phaff and *Rhodotorula creatinivorans* Golubev. In the D1D2 region of the 26S rRNA gene the base sequence of strain UG-20 was identical to that of the type strain of *L. scottii* (CBS 5930) but in the ITS region the closest species is *R. creatinivora* with only two base pairs different. Both species are very close relatives, differing in only one base pair in the D1D2 region and four base pairs in the ITS region. For this reason, *R. creatinivora* recently was renamed by Sampaio et al. (2003) as *Leucosporidiella creatinivora* (Golubev) Sampaio.

The predominance of some yeast species in the enrichment cultures in glucose urotropine medium did raise the question whether more yeast species are able to grow with urotropine as sole nitrogen source. For this reason part of the author's yeast culture collection was screened for this character in YCB broth as well as in GB glucose medium. In order to minimize spontaneous hydrolysis of urotropine, the pH of both

Table 1 Assimilation of urotropine by yeast strains and by some fungi and yeastlike fungi

Strains able to assimilate urotropine as sole nitrogen source in YCB and in GB medium at pH 6.5:
Ascomycetes
Candida ergatensis Santa Maria DoW-73 (CBS 10109, NRRL Y-27662)
Candida nitratophila (Shifrine et Phaff)Meyer et Yarrow CBS 2027^{T}
Candida paludigena Golubev et Blagodatskaya SpaW-71 (CBS 10108, NRRL Y-27666)
Candida saitoana Nakase et Suzuki TF-1
Candida shehatae Buckley et van Uden var. *insectosa* Kurtzman FaB-82 (CBS 10110, NRRL Y-27663)
Debaryomyces polymorphus (Klöcker)Price et Phaff PiB
Galactomyces geotrichum (Butler et Petersen) Redhead et Malloch RoW-72
Hansenula polymorpha Morais et Maia (syn. *Pichia angusta*) CBS 3742^{T}
Kazachstania bulderi (Middelhoven et al.)Kurtzman CBS 8638^{T}
Pichia capsulata (Wickerham) Kurtzman CBS 1993^{T}
Pichia henricii (Wickerham) Kurtzman CBS 2028
Pichia pastoris (Guillermond) Phaff CBS 704^{T}
Yarrowia lipolytica (Wickerham et al.) van der Walt et von Arx CBS 6124^{T}
Basidiomycetes
Cryptoccocus flavescens (Saito) C.E. Skinner CBS 942^{T}
Cryptococcus haglerorum Middelhoven et al. CBS 8902^{T}
Cryptococcus laurentii (Kufferath) C.E. Skinner CBS 139^{T}
Cryptococcus musci Takashima et al. FaB-83 (CBS 9492, JCM 13615)
Leucosporidium scottii Fell et al. CBS 5930^{T}
Trichosporon gamsii Middelhoven, Scorzetti et Fell CBS 8245^{T}, JCM 12197
Trichosporon porosum (Stautz)Middelhoven, Scorzetti et Fell BS-1
Trichosporon wieringae Middelhoven CBS 8903^{T}, JCM 12201
Strains able to assimilate urotropine in GB-glucose medium pH 6.5, but not in YCB:
Ascomycetes
Aureobasidium pullulans (de Bary) Arnaud FaW-15
Arxula adeninivorans (Middelhoven et al.)van der Walt et al. CBS 8244^{T}
Candida albicans (Robin) Berkhout BF-1
Candida bertae Ramírez et González BeW-2 (CBS 10112)
Candida bertae Ramírez et González FaW-12
Endomyces scopularis NRRL Y-17663^{T}
Mucor circinelloides f. *lusitanicus* (Bruderlein) Schipper S30P1
Pichia guillermondii Wickerham PiB-37
Schizoblastosporion starkeyi-henricii Ciferri NRRL Y-27344^{T}
Yarrowia lipolytica (Wickerham et al.) van der Walt et von Arx BeW-71
Basidiomycetes
Cryptococcus allantoinivorans Middelhoven CBS 9604^{T}, JCM 12200
Cryptococcus humicola (Daszewska)Golubev 5DG-302
Cryptococcus laurentii (Kufferath)C.E.Skinner CBS 7140, JCM 12199
Cryptococcus fagi Middelhoven et Scorzetti sp.nov. FaW-11 (CBS 9964^{T}, JCM 13614)
Guehomyces pullulans (Lindner) Fell et Scorzetti BJ-2, CBS 9201
Leucosporidiella creatinivora (Golubev)Sampaio CBS 8602^{T}
Papiliotrema bandonii Sampaio et al. CBS 9107^{T}
Rhodotorula mucilaginosa (Jörgensen)Harrison TA-3
Trichosporon asahii Akagi ex Sugita et al. TA-2
Trichosporon dehoogii Middelhoven, Scorzetti et Fell CBS 8686^{T}, JCM 12196
Trichosporon guehoae Middelhoven, Scorzetti et Fell CBS 8521^{T}
Trichosporon laibachii (Windisch) Guého et M.Th. Smith FaB-81
Trichosporon moniliiforme (Weigmann et Wolf) Guého et M.Th. Smith VL-4
Trichosporon smithiae Middelhoven, Scorzetti et Fell CBS 8370^{T}, JCM 12195
Trichosporon veenhuisii Middelhoven, Scorzetti et Fell CBS 7136^{T}
Trichosporon vadense Middelhoven, Scorzetti et Fell CBS 8901^{T}, JCM 12194
Strains unable to assimilate urotropine as sole nitrogen source:
Ascomycetes
Acremonium strictum W.Gams CBS 14253
Candida boleticola Nakase FaW-71 (CBS 10111, NRRL Y-27664)

Table 1 continued

Candida cretensis sp.nov. TF-2 (CBS 9453)
Candida vadensis sp.nov. BF-2 (CBS 9454)
Candida scorzettiae sp.nov. QuB-82 (CBS 10107, NRRL Y-27665)
Kodamaea ohmeri (Etchells et Bell) Yamada et al. CBS 9452
Basidiomycetes
Cryptococcus carnescens (Verona et Luchetti) Takashima et al. CBS 973^T
Cryptococcus nemorosus Golubev CBS 9606^T
Cryptococcus peneaus (Phaff et al.)Takashima et al. CBS 2409^T
Cryptococcus perniciosus Golubev CBS 9605^T
Cryptococcus terricola Pedersen Tan-2
Cryptococcus podzolicus (Bab'eva et Reshetova) Golubev BeB-82 (CBS 9491)
Rhodotorula cresolica Middelhoven et Spaaij CBS 7998^T
Trichosporon scarabaeorum Middelhoven, Scorzetti et Fell CBS 5601, JCM 12198

media was adjusted to pH 6.5 by addition of concentrated NaOH solution. The results are shown in Table 1.

Assimilation of urotropine as sole nitrogen source was a very common character, shown by many ascomycetes and basidiomycetes of various phylogenetic affiliation. In most cases growth was observed after one or two days, which excludes spontaneous hydrolysis of urotropine. In total 60 strains were examined, all belonging to different species, 28 of ascomycetous and 32 of basidiomycetous affiliation (Table 1). Of these, 13 ascomycetes, methylotrophic as well as non-methylotrophic species, and 8 basidiomycetes grew well in YCB-urotropine and in GB-glucose-urotropine broth. Only 6 of the ascomycetes and 8 of the basidiomycetes tested failed to grow in these media. Remarkably, 9 ascomycetes and 16 basidiomycetes assimilated urotropine in GB-glucose broth but not in YCB. Possibly, the high potassium phosphate concentration present in the former medium and in the enrichment culture favours assimilation of urotropine.

Urotropine supports slow growth of some soil-borne bacteria when supplied as sole source of carbon, nitrogen and energy. Pure cultures of *B. diminuta* and a *Phyllobacterium* sp. isolated from the crude enrichment culture also grew on urotropine but at a lower rate. The *Phyllobacterium* strain lost this character after subcultivation on rich media. Addition of glucose to the urotropine medium resulted in growth of some yeast species. Assimilation of urotropine as sole nitrogen source by yeasts is very common. Screening of a yeast culture collection revealed that 46 out of 60 species tested showed this characteristic. This is surprising as urotropine is not known to occur in nature. However, formation of trace amounts in the living cell is feasible as both formaldehyde and ammonia are normal metabolites. This could explain why so many yeast species are able to metabolize urotropine.

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