ORIGINAL PAPER

Harald Bothe · K. Møller Jensen · A. Mergel · J. Larsen
C. Jørgensen · Hermann Bothe · L. Jørgensen

Heterotrophic bacteria growing in association with *Methylococcus capsulatus* (Bath) in a single cell protein production process

Abstract The methanotrophic bacterium *Methylococcus capsulatus* (Bath) grows on pure methane. However, in a single cell protein production process using natural gas as methane source, a bacterial consortium is necessary to support growth over longer periods in continuous cultures. In different bioreactors of Norferm Danmark A/S, three bacteria consistently invaded *M. capsulatus* cultures growing under semi-sterile conditions in continuous culture. These bacteria have now been identified as a not yet described member of the *Aneurinibacillus* group, a *Brevibacillus agri* strain, and an acetate-oxidiser of the genus *Ralstonia*. The physiological roles of these bacteria in the bioreactor culture growing on natural, non-pure methane gas are discussed. The heterotrophic bacteria do not have the genetic capability to produce either the haemolytic enterotoxin complex HBL or non-haemolytic enterotoxin.

Introduction

Norferm Danmark A/S has developed BioProtein, a bacterial single cell protein (SCP) product that serves as a protein source in feedstuff. The culture mainly consists of the methanotrophic bacterium, *Methylococcus capsulatus* (Bath). Methanotrophs can utilise methane as a sole source of carbon and energy (reviewed by Hanson and Hanson 1996). The first step in methane conversion is the oxidation of methane to methanol catalysed by methane monooxygenase (MMO). Two types of MMOs are known: a cytoplasmic or soluble form (sMMO) and a membrane-bound or particulate enzyme (pMMO). High copper/cell ratios in the culture favour pMMO synthesis, whereas sMMO is formed at low copper/biomass ratios (Stanley et al. 1983). When *M. capsulatus* is used for SCP production, pMMO formation is preferred because this enzyme allows a more efficient carbon to biomass conversion than sMMO (Jørgensen and Degn 1987).

Besides methane, both sMMO and pMMO oxidise several other substrates (Colby and Dalton 1978; Stirling and Dalton 1979; Dalton 1981). Generally, pMMO exhibits a narrower substrate spectrum than sMMO. pMMO only catalyses the oxidation of unbranched alkanes and alcenes of up to five C-atoms (Hou et al. 1980; Burrows et al. 1984), and mainly ethane, but also propane, can serve as alternative substrates to methane oxidation (Carlsen 1992). Reaction products of pMMO, such as ethanol or propanol, can be further oxidised by the subsequent enzymes of the methane oxidation pathway, i.e. methanol dehydrogenase and formaldehyde dehydrogenase, leading to the accumulation of carboxylic acids in the bioreactor (Leadbetter and Forster 1958; Linton and Drozd 1982). Such acids and other possible oxidation products can cause toxicity in the bioreactor (Sullivan et al. 1998). Acetate, for example, inhibits growth of *M. capsulatus* (Zhivotchenko et al. 1989).

Depending on the gas field, natural gas contains different concentrations of ethane (2.7–20.0%), propane (0.4–13.0%) and higher alkanes (0.1–6.1%; Linton and Drozd 1982). Hence, acetate and propionate accumulate in the production plant. One solution to overcome toxic levels of these carboxylic acids is to establish a stable mixed culture with heterotrophic bacteria (Wilkinson et al. 1974).

In various experiments performed at different bioreactors of Norferm Danmark A/S, three different bacteria were repeatedly found to invade *M. capsulatus* cultures growing under semi-sterile conditions. These bacteria were isolated and characterised as a Gram-negative

Harald Bothe (✉) · K. Møller Jensen · J. Larsen · C. Jørgensen
Hermann Bothe · L. Jørgensen
Norferm Danmark A/S, Stenhuggervej 22,
5230 Odense M, Denmark

A. Mergel · Hermann Bothe
Botanisches Institut, Universität zu Köln, Gyrhofstrasse 15,
50923 Cologne, Germany

Present address:
Harald Bothe, Institut für Lebensmitteltechnologie,
Universität Hohenheim, Emil-Wolff-Strasse 14,
70599 Stuttgart, Germany,
e-mail: hbothe@Uni-Hohenheim.de,
Tel.: +49-711-4592313, Fax: +49-711-4594276

acetate oxidiser (termed "DB3") and two Gram-positive, spore-forming rods ("DB4" and "DB5"). These three heterotrophic strains represented typically less than 2% of the total bacterial population of semi-sterile *M. capsulatus* cultures growing on pure methane. However, cultures growing on a mixture of methane and ethane (95%/5%) contained up to 13% heterotrophs. The percentage of each heterotroph varied considerably depending on the gas mixture, but DB3 was always the dominant bacterium. When grown in continuous culture on natural gas (composed of approximately 91% methane, 5% ethane, 1.7% propane, 1% butane and traces of higher alkanes; supplied by Naturgas Fyn, Odense, Denmark), the following composition was observed: 80% *M. capsulatus*, 19% DB3, 0.3% DB4 and 0.5% DB5. *M. capsulatus* did not grow for longer periods (i.e. 2–3 days) on natural gas in the absence of the heterotrophs. A detailed characterisation of these bacteria is described in the present communication.

Since BioProtein serves as feedstuff, the formation of toxins by the heterotrophs has to be ruled out. Some *Bacillus* isolates, namely strains of *B. cereus*, are known to produce several enterotoxins. The haemolytic enterotoxin complex HBL is probably the primary virulence factor in *B. cereus* diarrhoea (Beecher et al. 1995) and the capability to form HBL is widely distributed in the *B. cereus* group (Prüß et al. 1999). Additionally, a non-haemolytic enterotoxin (NHE) is involved in food poisoning (Lund and Granum 1996, 1997). The availability of the genes *nhe* and *hbl*, coding for these toxins, allows PCR-based identification using published primers annealing to conserved regions (Heinrichs et al. 1993; Ryan et al. 1997; Granum et al. 1999).

Materials and methods

Isolation and growth of bacteria

M. capsulatus (Bath) NCIMB 11132 was grown in nitrate/mineral salts (NMS) medium as described by Larsen and Jørgensen (1996). Strains DB3, DB4 and DB5 were repeatedly isolated from *M. capsulatus* cultures growing in continuous culture on a mixture of 95% methane and 5% ethane (v/v); these strains were plated out on Plate Count Agar (Difco). Pure cultures of *B. cereus* CCUG 7414, DB3, DB4 and DB5 were grown in 50 ml yeast extract (20 g/l) at 45°C with shaking at 200 rpm.

Cloning and sequencing of 16S rRNA genes

Genomic DNA was prepared from liquid overnight cultures of DB3 and DB5 and from a 2-day-old liquid culture of DB4 by the method of Marmur (1961). For PCR experiments, a DNA thermal cycler (Biometra), *Taq* polymerase (Promega, Madison, Wis.) and primers (synthesised by MWG Biotech, Ebersberg, Germany) were used. PCR amplification of a 1.5 kb segment of the 16S rRNA gene of DB3 was carried out using primers RM 1 (5′-AGAGTTTGATCMTGGCTCAGAWTR-3′) and "reverse 1" (5′-ACGGYTACCTTGTTACGACTT-3′). From the DB4 16S rRNA gene, a 700 bp segment of the 5′-end was amplified by using primers RM 1 and RM 2 (5′-GA CTCTACGCATTTCACC-GCTAC-3′). In the case of the 16S rRNA gene of DB5, three individual PCR products were obtained: a 350 bp segment using

primers forward non-EUB 338 (Wallner et al. 1993) and RM 2, a 1.35 kb product using primers RM 1 and "reverse 2" (5′-TGCTGATCCGCGATTACTAGC-3′), and a 1.5 kb product using primers RM 1 and "reverse 1". For the amplification of 350 or 700 bp PCR products, the following 30 cycles were carried out: 20 s, 94°C; 45 s, 50°C and 60 s, 72°C. To obtain 1.35 or 1.5 kb products, the following 30 cycles were used: 20 s, 94°C; 60 s, 50°C and 90 s, 72°C. The PCR products were purified using a Qiaquick DNA purification kit (Qiagen, Hilden, Germany). DNA was then ligated into the sequencing vector pGEM T-Easy (Promega, Madison, Wis.). *Escherichia coli* XL1-blue (Stratagene, The Netherlands) was transformed with the ligation mixtures. Plasmid DNA was isolated from *E. coli* clones by the alkaline lysis method (Birnboim and Doly 1979). Sequencing was performed on an ABI PRISM 337 DNA Sequencer (PE Applied Biosystems, Weiterstadt, Germany). The PCR products amplified from the DNA of DB3 and DB5 16S rRNA genes were sequenced three times and the DB4 PCR segment was sequenced twice. The data were analysed using the database of the NCBI (USA).

PCR assay for enterotoxin genes *nhe* and *hbl*

A liquid overnight batch culture of *B. cereus* CCUG 7414 was used to prepare genomic DNA (Marmur 1961). Approximately 50 ng of each *B. cereus*, DB3, DB4 and DB5 genomic DNA was used as PCR template. The sequences of primers (50 pmol each) for the amplification of the *nheB-nheC* genes were: 5′-CGGTT-CATCTGTTGCGACAGC-3′ and 5′-CGACTTCTGCTTGTGCT-CCTG-3′ (Granum et al. 1999). Primers for the amplification of the *hblD-hblA* genes were: 5′-CGCTCAAGAACAAAAAGTAGG-3′ and 5′-CTCCTTGTAAATCTGTAATCCCT-3′ (Heinrichs et al. 1993). The 30 PCR cycles were: 20 s, 94°C followed by 60 s, 50, 53 or 55°C and finally 90 s, 72°C.

PCR identification of strains belonging to the *Brevibacillus* and the *Aneurinibacillus* groups

A segment of the 16S rRNA gene of DB5 was amplified with primers BREV174 and 1377R following a published protocol (Shida et al. 1996). A 16S rRNA gene specific segment of DB4 was obtained with primers ANEU506F (position 519 was changed to A) and 1377R (Shida et al. 1996).

Biochemical and microbiological assays

Assimilation of citrate was tested with Simmon's Citrate Agar (Difco). For Gram-staining, bacteria were grown for 24 h on Nutrient Agar (Difco), suspended in 3% KOH for lysis of Gram negative cells (Gregersen 1978) and stained using the Difco kit. Spore formation was tested with a suspension (in 0.9% NaCl) of cells grown on Nutrient Agar at 45°C and heat-treated for 15 min at 85°C (Claus and Berkeley 1986). Tests for acid formation from fructose and mannitol (10 g/l) were performed in tubes with OF basal medium (Difco). Acid formation from ethanol (1% v/v) was tested in slanted tubes with aerobic low peptone medium (Hendrickson and Krenz 1991). Urease activity was tested on Christensen's Urea Agar (Oxoid). The Voges-Proskauer reaction was performed by adding 3 ml of 40% NaOH and 0.6 g creatine to 5 ml V-P-bouillon after 5 days of incubation at 45°C (Claus and Berkeley 1986). Catalase activity was determined as described by Claus and Berkeley (1986). For further identification, the API 50 CHB test (BioMerieux, France) was used at 45°C. Formic, acetic, propionic, and butyric acids (all 0.1%, w/v), alanine and glycine (both 0.1 M) were assayed as growth substrates in NMS medium (Whittenbury et al. 1970). DNA was quantified by the diphenyl-amine method (Herbert et al. 1971), RNA by the orcinol method (Herbert et al. 1971) and proteins using a BioRad kit (BioRad, Munich, Germany). Total organic carbon (TOC) was measured using a Shimadzu TOC-5000 analyser.

Table 1 Sequence identities of the DNA coding for the 16S rRNA gene of DB3. The numbers in brackets represent the position of the bases compared with the DB3 sequences. The 5′-end of the *Ralstonia gilardii* LMG 5886 gene contains a number of unidentified bases lowering the sequence identity

16S rRNA of	5′-end (1–487) (%)	3′-end (939–1489) (%)
Ralstonia sp. PHS 1	98.5	98.7
Burkholderia sp. C37KA	97.5	98.8
R. gilardii LMG 5886	94.0	98.5
R. eutropha JS 705	95.9	98.1
R. paucula LMG 3413	95.7	98.2

Table 2 Identities of a 700 bp sequence coding for the 5′-end of the DB4 16S rRNA gene

16S rRNA of	Similarity to the DB4 sequence (%)
Bacillus aneurinolyticus DSM 5562	97.0
Bacillus migulanus DSM 2895	96.8
Aneurinibacillus migulanus ATCC 9999	96.7
Bacillus acidovorans ATCC 51159	96.3
Aneurinibacillus aneurinolyticus NCIMB 10056	95.9
Bacillus thermoaerophilus DSM 10154	94.3

Strain DB3 has been deposited with the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland, as "*Alcaligenes acidovorans*" NCIMB 13287. Strain DB4 has been deposited as NCIMB 13288 and strain DB5 as NCIMB 13289.

The GenBank sequence accession numbers for strain DB3 are AF369868 (5′-end of the putative 16S rRNA gene sequence) and AF369869 (3′-end). The partial 16S rRNA gene sequence from strain DB4 has the GenBank accession number AF369870.

Results

Molecular biological identification of DB3, DB4, and DB5

To identify DB3, the 5′- and 3′-ends of a 1.5 kb PCR product of the 16S rRNA gene were sequenced. The sequence shares strong identities with 16S rRNA genes from various *Ralstonia* strains (Table 1), especially with strain PHS 1 (Lee and Lee 2000).

For DB4, the sequence of a 16S rRNA gene specific segment starting at the 5′-end and totalling 699 nucleotides was determined. The sequence was 94–97% identical to 16S rRNA sequences of members of the *Aneurinibacillus* cluster (Shida et al. 1996, see Table 2). A variation equal to 2% between two sequences is generally interpreted that an isolate (in this case DB4) is not identical to any other organism deposited so far (Fox et al. 1992). The identities of around 95% are, however, strong enough to assign this bacterium to the *Aneurinibacillus* cluster. Members of this cluster share less than 91.3% identity with species from other clusters (Shida et al. 1996).

When a PCR protocol including a specific primer to identify members of the *Aneurinibacillus* group (Shida et

Table 3 Sequence identities of the DNA coding for the DB5 16S rRNA gene. The numbers in brackets represent the position of the bases compared with the DB5 sequences. The first 44 bp from the 5′-end and the last 90 bp towards the 3′-end were omitted because not all the sequences available from the database are complete at these positions

16S rRNA of	5′-end (45–700) (%)	3′-end (850–1,410) (%)
Brevibacillus agri NRRL NRS-1219	99.1	99.0
Bacillus agri NRRL NRS-1689	99.5	99.0
Brevibacillus formosus NRRL NRS-863	97.5	99.7
Brevibacillus reuszeri NRRL NRS-1206	97.5	98.8
Brevibacillus choshiensis HPD 52	97.4	98.8
Brevibacillus sp. Riau	98.0	98.4
Brevibacillus parabrevis IFO 12334	96.8	99.1
Brevibacillus brevis JCM 2503	95.8	99.1
Bacillus brevis NCIMB 9372	96.5	–
Brevibacillus centrosporus NRRL NRS-664	94.3	99.1
Brevibacillus sp. 96452	93.5	99.1
Brevibacillus borstelensis NRRL NRS-818	94.8	97.4
Bacillus laterosporus NCDO 1763	91.1	98.4

al. 1996) was applied, a PCR segment of the expected size (ca. 900 bp) was obtained. No PCR product was generated from DB5 when the same primers and conditions were used.

For DB5, three individual PCR products of 0.35, 1.35 and 1.5 kb were used to determine the sequence of the 16S rRNA gene. The available information covers the first 700 nucleotides from the 5′-end of the gene, followed by a gap of 145 nucleotides. The residual bases towards the 3′-end were also sequenced. The analysed parts of the DB5 gene are more than 99% identical to *Brevibacillus agri* NRRL NRS-1219 and *Bacillus agri* NRRL NRS-1689 indicating that DB5 is a *Brevibacillus agri* strain (Shida et al. 1996). High homologies to other strains of the *Brevibacillus* group were found (Table 3). Using specific primers for identifying members of the *Brevibacillus* cluster (Shida et al. 1996), a PCR segment of the expected size (ca. 1.2 kb) was amplified from genomic DNA of DB5. No PCR product was detected when genomic DNA from DB4 served as template.

Morphological and biochemical characteristics of DB3, DB4, and DB5

DB3 (Ralstonia sp.)

When grown on nutrient agar for 48 h at 45°C, strain DB3 formed shiny, cream-coloured colonies of 2–3 mm diameter. The morphology of the colonies varied from circular with a smooth surface and entire edge to irregular with a rough surface. The colonies contained straight rods with a diameter of 0.7 μm and a length of 2.0–3.0 (exceptionally 5.0) μm. Cells were motile by means of peritrichous flagella. Gram-staining and electron transmission microscopy showed that DB3 is Gram-negative. The isolate did not form spores, and no viable cells were

Table 4 Analysis of supernatant fractions from a pure methane-grown continuous culture of *Methylococcus capsulatus* before and after 48 h of incubation at 45°C. Bacteria (strains DB3, DB4 or DB5) were added to some of the fractions. The lowest line displays colony-forming units (cfu) at the end of the incubation; cfu were measured on Plate Count Agar after incubation at 45°C for another 48 h

	Before incubation Supernatant	After incubation			
		Supernatant – bacteria	Supernatant + DB3	Supernatant + DB4	Supernatant + DB5
Total organic carbon (TOC) (ppm)	189	196	157	161	133
Protein (ppm)	118	58	59	64	66
Free amino acids (ppm)	34	56	5	7	1
RNA (ppm)	62	75	80	68	68
DNA (ppm)	12	12	13	12	6
Acetate (ppm)	6	6	0	0	4
Cfu/ml	$<10^1$	$<10^1$	5.0×10^7	1.3×10^7	3.8×10^6

present in a cell suspension heat-treated for 15 min at 85°C.

The bacterium DB3 was catalase, oxidase and Voges-Proskauer positive. It did not synthesise indole and did not form ornithine decarboxylase, β-galactosidase, urease, lysine decarboxylase, and tryptophan deaminase. It hydrolysed Tween 80 and gelatine, but not aesculin and did not produce acid from glucose. Neither reduction of NO_3^- to NO_2^- or N_2 nor reduction of SO_4^{2-} to H_2S was observed under anaerobic incubation. DB3 grew on yeast extract (3 g l^{-1}) at pH values from 5.4 to 9.1 and the temperature optimum was 47°C.

DB4 (Aneurinibacillus sp.)

Colonies of this isolate had a diameter of 4–6 mm when grown on nutrient agar for 24 h at 45°C. They had a dome-shaped form with white edges, smooth surface and a creamy consistency. In transmission light microscopy, they appeared dark because of the high content of ellipsoid spores of 0.5×1 µm within the colonies. These also contained straight rods of a diameter of 1–1.5 µm and a length of 3–8 µm. Strain DB4 was Gram-positive and non-motile under the growth conditions employed.

The isolate DB4 was catalase and oxidase positive, Voges-Proskauer negative and hydrolysed gelatine. It did not produce ornithine decarboxylase, β-galactosidase, indole, urease, lysine decarboxylase, arginine dihydrolase or tryptophan deaminase. The isolate did not produce acid from glucose. It reduced NO_3^- to NO_2^- and NO_2^- to N_2, but not SO_4^{2-} to H_2S.

DB5 (Brevibacillus agri)

After 24 h at 45°C on nutrient agar, DB5 colonies had a diameter of 2–4 mm. They were flat, circular and yellowish with a rough surface and uneven edge and contained motile rods of 0.8–1.0×2–6 µm that tested Gram-positive. Spores, which had a cylindrical shape of 0.5–0.7×1.5–2.5 µm, were occasionally observed. The isolate DB5 was catalase, oxidase and Voges-Proskauer negative. It synthesised arginine dihydrolase, but not ornithine decarboxylase, β-galactosidase, indole, urease, lysine decarboxylase or tryptophan deaminase. The cells hydrolysed gelatine and produced acid from glucose. They did not form NO_2^- from NO_3^- or H_2S from SO_4^{2-}.

The three isolates remove organic compounds from *M. capsulatus* cultures

The three isolates were tested for their capacity to grow on organic matter released by a *M. capsulatus* culture. A cell-free supernatant fraction from a pure continuous methane-grown culture of *M. capsulatus* was incubated for 48 h at 45°C. Afterwards, the fraction contained approximately half of the original protein concentration, whereas the amino acid content was significantly increased (Table 4). This suggests that *M. capsulatus* proteases degrade proteins present in the supernatant. When the heterotrophic isolates DB3, DB4 and DB5 were added separately to parallel supernatant aliquots, a substantial reduction of amino acids (from 34 to 7 ppm) was observed after the incubation period, indicating that all three strains utilised amino acids. The amount of protein was not significantly altered in any of the experiments compared to the reference incubation without bacteria, showing that the heterotrophs did not utilise whole protein. The RNA content was also unaltered, whereas DB5 degraded DNA (from 12 to 6 ppm). DB3 and DB4 were observed to utilise acetate completely. Total organic carbon was most effectively reduced by DB5 (from 190 to 133 ppm), and DB3 and DB4 were also able to minimise TOC (both to a level of ca. 160 ppm).

The growth of the isolates on acetate was investigated in another set of batch culture experiments. In NMS (Larsen and Jørgensen 1996) with 0.9% (w/v) acetate as sole carbon source, DB3 grew at a rate, $\mu =0.60$ h^{-1} to a yield, $Y =0.43$ g biomass/g acetate during exponential growth. The data for DB4 are: $\mu =0.58$ h^{-1} and $Y =0.40$ g biomass/g acetate. DB5 grew at $\mu =0.45$ h^{-1} to $Y =0.30$ g biomass/g acetate.

PCR-based assay for enterotoxins

In PCR experiments, primers designed for the amplification of segments of the toxicity genes *nhe* (Granum et al. 1999) and *hbl* (Heinrichs et al. 1993; Ryan et al. 1997) were tested. Using genomic DNA isolated from *B. cereus*

as a positive control, an *nhe*-specific PCR product of 1.45 kb and an *hbl*-specific segment of 1.65 kb were amplified. No PCR products were obtained when genomic DNA of DB4 or DB5 was used as template even under low stringencies (annealing temperature =50°C).

Discussion

Identification of the three heterotrophic strains

A mixed culture is necessary when methanotrophic bacteria are used for the production of SCP from natural gas (Drozd and McCarthy 1981). In the BioProtein culture, three heterotrophic strains grow in association with *M. capsulatus*. In the present study, these were identified as *Brevibacillus agri*, *Ralstonia* sp. and *Aneurinibacillus* sp. by sequencing parts of their 16S rRNA genes. The iden-

tification of the two *Bacillus* strains was confirmed by a PCR method using primers specific for the detection of members of the *Aneurinibacillus* and *Brevibacillus* groups (Shida et al. 1996). The association of *M. capsulatus* with the three heterotrophs was observed in various BioProtein production reactors. The strains are nowadays added to the *M. capsulatus* culture to facilitate its growth. It can be assumed that very similar strains will invade an *M. capsulatus* culture when this organism is grown on natural gas at other locations. It is noteworthy in this context that the sequence of a *Ralstonia* sp. PHS1 isolate recently deposited at the NCBI databank (Lee and Lee 2000) shows 98% identity to the *Ralstonia* strain characterised here (see Table 1). Lee and Lee (2000) describe this Korean isolate as a novel thermotolerant, versatile bacterium that degrades benzene, toluene, ethylbenzene and *o*-xylene.

Isolate DB3 also shows strong 16S rRNA sequence identity to the well-described *Ralstonia gilardii* type strain LMG 5886 (Table 1). Additionally, the phenotypic characteristics coincide with typical features described for *R. gilardii* strains (Coenye et al. 1999, see Table 5). These features can be used to discriminate *R. gilardii* from other *Ralstonia*-species, such as *R. eutropha*, *R. pickettii* and *R. solanacearum* (Coenye et al. 1999).

The biochemical characterisation of the two *Bacillus* isolates is in accord with the molecular biological data, but some dissimilarities are also obvious (Table 6). Nonswollen sporangia seen in the case of the newly isolated *B. agri* strain (DB5) are atypical for the *Brevibacillus* cluster. However, sporulation was not easily achieved under the culture conditions tested. Under the culture conditions employed, DB4 is non-motile, in contrast to all the members of the *Aneurinibacillus* group. Otherwise, this isolate exhibits typical features described for *A. aneurinolyticus* and *A. migulanus* strains (Shida et al. 1994a). These comprise bacteria of rod-shaped appearance which are Gram-positive and possess oval spores occurring in swollen sporangia.

Table 5 Biochemical characteristics of isolate DB3 in comparison with *R. gilardii* LMG 5886

Test		DB3	*R. gilardii* LMG 5886[a]
Catalase		+	+
Oxidase		+	+
Urease		–	–
Growth at 42°C		+	+
$NO_3^- \rightarrow NO_2^-$		–	–
Assimilation of	D-Glucose	–	–
	D-Galactose	–	–
	p-Hydroxybenzoate	–	–
	D-Fructose	–	–
	2-Ketogluconate	–	–
	D-Malate	+	+
	L-Arabinose	–	–
	n-Caprate	+	–
	D-Fucose	–	–
	D-Xylose	–	–
	N-Acetylglucosamine	–	–

[a] Data from Coenye et al. (1999)

Table 6 Characteristics of the two *Bacillus* strains DB4 and DB5 in comparison with *A. migulanus* ATCC 9999 and *Brevibacillus agri* NRRL NRS-1219

Test		DB4	*A. migulanus* ATCC 9999[a]	DB5	*B. agri* NRS-1219
Catalase		+	+	–	+[b]
Oxidase		+	+	–	–[b]
$NO_3^- \rightarrow NO_2^-$		+	+	–	–[b]
Sporangia		Swollen	Swollen	Not swollen	Swollen[b]
Cell size		1–1.5×3–8 µm	0.5–1×2–6 µm	0.8–1×2–6 µm	0.5–1×2–5 µm[b]
Acid from	D-Glucose	–	–	+	+[b]
	L-Arabinose	–	–	–	–[b]
	D-Xylose	–	–	–	–[c]
	D-Mannitol	–	–	+	+[b]
Hydrolysis of	Gelatine	+	–	+	+[c]
	Starch	–	–	–	–[b]
	Tween 80	–	–	+	+[c]
Utilisation of citrate		–	–	–	–[b]
Voges-Proskauer		–	–	–	–[b]
Motility		–	+	+	+[b]
Gram-staining		+	+	+	+[b]/±[c]
Growth at 50°C		+	+	+	max. 40°C[b]; +[c]

[a] Data for *Bacillus migulanus* = *Aneurinibacillus migulanus* from Takagi et al. (1993)

[b] Data for *Bacillus agri* NRLL NRS-1219 from Nakamura (1993)

[c] Data for *Bacillus galactophilus* NRRL NRS-616 from Takagi et al. (1993). *B. galactophilus* was later identified as *Bacillus agri* (Shida et al. 1994b)

The role of the three heterotrophic strains
in the fermentation process

The heterotrophic strains eliminate organic carbon from the bioreactor. The main role of *Ralstonia* sp. very likely consists of removing acetate (and possibly other carboxylic acids) formed by *M. capsulatus*. The two *Bacillus* strains also utilise acetate, but they exhibited poorer growth yields than the *Ralstonia* isolate in batch culture experiments. Without *Ralstonia* sp., it is in fact difficult (if not impossible) to establish a bioreactor cultivation of the mixed BioProtein culture on natural gas or methane containing 5% ethane. In such cultivations, acetate accumulated to inhibitory concentrations (470–475 ppm), whereas lower acetate concentrations (225 ppm) were observed when *M. capsulatus* was grown in co-culture with only the *Ralstonia* strain.

The bacilli are involved in elimination of further organic material. The *Brevibacillus agri* strain especially, utilised organic matter effectively and was the only isolate which degraded DNA. None of the bacteria utilised whole proteins, but once these were degraded to smaller peptides or free amino acids (probably by *M. capsulatus* proteases), all isolates removed these from the broth.

Because of the limited amount of organic matter in the BioProtein culture, the risk of contamination by other (possibly pathogenic) bacteria is marginal. An industrial scale SCP production process is not feasible under completely sterile conditions. Any contamination, especially by *Bacillus cereus* strains known to cause food poisoning (Lund 1990) has to be avoided. Both the *Aneurinibacillus* and the *Brevibacillus agri* strains were found to be taxonomically distinct from the *Bacillus cereus* group. Additionally, PCR experiments indicated that both *Bacillus* strains do not possess genes encoding the enterotoxins NHE and HBL. Clearly, negative results in PCR experiments have to be interpreted cautiously. However, the toxicity genes are very much conserved among different bacteria (Prüß et al. 1999) and control experiments with bacteria possessing the genes were positive. Thus, all the data obtained in the present study indicate that the contaminants are non-toxic, which is a prerequisite for their use in the BioProtein culture.

It may be argued that the co-culture of *M. capsulatus* (Bath) with the now identified *Ralstonia*, *Aneurinibacillus* and *Brevibacillus agri* strains is a special case during BioProtein production. However, natural gas as a cheap methane source contains a number of organic compounds that prevent growth of *M. capsulatus*. Consequently, a consortium of heterotrophic bacteria like the one described in the present study will always be necessary to degrade these compounds and thus enable growth of methanotrophic bacteria on natural gas.

References

Beecher DJ, Schoeni JL, Wong ACL (1995) Enterotoxin activity of haemolysin BL from *Bacillus cereus*. Infect Immun 63: 4423–4428

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1518

Burrows KJ, Cornish A, Scott D, Higgins IJ (1984) Substrate specificities of the soluble and particulate methane mono-oxygenases of *Methylosinus trichosporium* OB3b. J Gen Microbiol 130:3327–3333

Carlsen HN (1992) Applications of stopped flow membrane inlet mass spectrometry in microbial physiology. Ph.D. Thesis, Odense University

Claus D, Berkeley RCW (1986) Genus *Bacillus*. In: Sneath PHA, Mair NS, Sharp ME (eds) Bergey's manual of systematic bacteriology, vol 2. Williams and Wilkins, Baltimore, pp 1105–1139

Coenye T, Falsen E, Vancanneyt M, Hoste B, Govan JRW, Kersters K, Vandamme P (1999) Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. Int J Syst Bacteriol 49:405–413

Colby J, Dalton H (1978) Resolution of the methane monooxygenase of *Methylococcus capsulatus* (Bath) into three components. Biochem J 171:461–468

Dalton H (1981) Methane mono-oxygenases from a variety of microbes. In: Dalton H (ed) Microbial growth on C1 compounds. Heyden, London, pp 1–10

Drozd JW, McCarthy PW (1981) Mathematical model of microbial hydrogen oxidation. In: Dalton H (ed) Microbial growth on C1 compounds. Heyden, London, pp 360–369

Fox GE, Wisotzekey JD, Jurtshuk P (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int J Syst Bacteriol 42:166–170

Granum PE, O'Sullivan K, Lund T (1999) The sequence of the non-haemolytic enterotoxin operon from *Bacillus cereus*. FEMS Microbiol Lett 177:225–229

Gregersen T (1978) Rapid method for distinction of Gram-negative from Gram-positive bacteria. Eur J Appl Microbiol Biotechnol 5:123–127

Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol Rev 60:439–471

Heinrichs JH, Beecher DJ, MacMillan JM, Zilinskas BA (1993) Molecular cloning and characterization of the *hblA* gene encoding the B component of haemolysin BL from *Bacillus cereus*. J Bacteriol 157:6760–6766

Hendrickson DA, Krenz MM (1991) Reagents and stains. In: Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ (eds) Manual of clinical microbiology. American Society for Microbiology, Washington, D.C. pp 1289–1314

Herbert D, Phipps PJ, Strange RE (1971) Chemical analysis of microbial cells. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 5. Academic Press, London, pp 209–334

Hou CT, Patel RN, Lanskin AI (1980) Epoxidation and ketone formation by C1-utilizing microbes. Adv Appl Microbiol 26: 199–202

Jørgensen L, Degn H (1987) Growth rate and methane affinity of a turbidostatic and oxystatic continuous culture of *Methylococcus capsulatus* (Bath). Biotechnol Lett 9:71–76

Larsen J, Jørgensen L (1996) Reduction of RNA and DNA in *Methylococcus capsulatus* by endogenous nucleases. Appl Microbiol Biotechnol 45:137–140

Leadbetter ER, Forster JW (1958) Studies on some methane-utilizing bacteria. Arch Microbiol 30:91–118

Lee SK, Lee SB (2000) Isolation and characterization of a novel thermotolerant bacterium that degrades benzene, toluene, ethylbenzene, and *o*-xylene, NCBI Databank, Accession no. 7578861

Linton JD, Drozd JW (1982) Microbial interactions and communities in biotechnology. In: Bull AT, Slater JH (eds) Microbial interactions and communities, vol 1. Academic Press, London, pp 357–406

Lund BM (1990) Foodborne disease due to *Bacillus* and *Clostridium* species. Lancet 336:982–986

Lund T, Granum PE (1996) Characterisation of a non haemolytic enterotoxin complex from *Bacillus cereus* after a foodborne outbreak. FEMS Microbiol Lett 141:151–156

Lund T, Granum PE (1997) Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*. Microbiology 143:3329–3336

Marmur J (1961) A procedure for isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:208–218

Nakamura LK (1993) DNA relatedness of *Bacillus brevis* Migula 1900 strains and proposal of *Bacillus agri* sp. nov. nom. rev. and *Bacillus centrosporus* sp. nov. nom. rev. Int J Syst Bacteriol 43:20–25

Prüß BM, Dietrich R, Nibler B, Märtlbauer E, Scherer S (1999) The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. Appl Environ Microbiol 65:5436–5442

Ryan PA, Macmillan JM, Zilinskas BA (1997) Molecular cloning and characterization of the genes encoding the L_1 and L_2 components of hemolysin BL from *Bacillus cereus*. J Bacteriol 179:2551–2556

Shida O, Takagi H, Kadowaki K, Yano H, Abe M, Udaka S, Komagata K (1994a) *Bacillus aneurinolyticus* sp. nov. nom. rev. Int J Syst Bacteriol 44:143–150

Shida O, Takagi H, Kadowaki K, Udaka S, Komagata K (1994b) *Bacillus galactophilus* is a later subjective synonym of *Bacillus agri*. Int J Syst Bacteriol 44:172–173

Shida O, Takagi H, Kadowaki K, Komagata K (1996) Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. Int J Syst Bacteriol 46:939–946

Stanley SH, Prior SD, Leak DJ, Dalton H (1983) Copper stress underlies the fundamental change in intra-cellular location of methane monooxygenase in methane-oxidizing organisms: studies in batch and continuous cultures. Biotechnol Lett 5:487–492

Stirling DI, Dalton H (1979) Properties of the methane monooxygenase from extracts of *Methylosinus trichosporium* OB3b and evidence for its similarity to the enzyme from *Methylococcus capsulatus* (Bath). Eur J Biochem 96:205–212

Sullivan JP, Dickinson D, Chase HA (1998) Methanotrophs, *Methylosinus trichosporium* OB3b, sMMO, and their application to bioremediation. Crit Rev Microbiol 24:335–373

Takagi H, Shida O, Kadowaki K, Komagata K, Udaka S (1993) Characterisation of *Bacillus brevis* with descriptions of *Bacillus migulanus* sp. nov. *Bacillus choshinensis* sp. nov. *Bacillus parabrevis* sp. nov. *and Bacillus galactophilus* sp. nov. Int J Syst Bacteriol 43:221–231

Wallner G, Amann R, Beisker W (1993) Optimizing fluorescent in situ hybridisation with rRNA-targeted oligonucleotide probes for cytometric identification of microorganisms. Cytometry 14:136–143

Whittenbury R, Phillips KC, Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61:205–218

Wilkinson TG, Topiwala HH, Hamer G (1974) Interactions in a mixed bacterial population growing on methane in continuous culture. Biotechnol Bioeng 16:41–59

Zhivotchenko AG, Davidov ER, Davidova EG, Rachinskii VV (1989) Effect of ethane-oxidation products on methane assimilation by *Methylococcus capsulatus*. Microbiology 58:591–594