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An obligate methylotrophic, methane-oxidizing *Methylomicrobium* species from a highly alkaline environment

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Abstract A new, obligately methylotrophic, methane-oxidizing bacterium, strain AMO 1, was isolated from a mixed sample of sediments from five highly alkaline soda lakes (Kenya). Based on its cell ultrastructure and high activity of the hexulose-6-phosphate synthase, the new isolate belongs to the type I methanotrophs. It differed, however, from the known neutrophilic methanotrophs by the ability to grow and oxidize methane at high pH values. The bacterium grew optimally with methane at pH 9–10. The oxidation of methane, methanol, and formaldehyde was optimal at pH 10, and cells were still active up to pH 11. AMO 1 was able to oxidize ammonia to nitrite at high pH. A maximal production of nitrite from ammonia in batch cultures at pH 10 was observed with 10% of CH_4 in the gas phase when nitrate was present as nitrogen source. Washed cells of AMO 1 oxidized ammonia most actively at pH 10–10.5 in the presence of limiting amounts of methanol or CH_4. The bacterium was also capable of oxidizing organic sulfur compounds at high pH. Washed cells grown with methane exhibited high activity of CS_2 oxidation and low, but detectable, levels of DMS and DMDS oxidation. The GC content of AMO 1 was 50.9 mol%. It showed only weak DNA homology with the previously described alkaliphilic methanotroph "*Methylobacter alcaliphilus*" strain 20Z and with the neutrophilic species of the genera *Methylobacter* and *Methylomonas.* According to the 16*S* rRNA gene sequence analysis, strain AMO 1 was most closely related to a neutrophilic methanotroph, *Methylomicrobium pelagicum* (98.2% sequence similarity), within the gamma-*Proteobacteria*.

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Introduction

Alkaliphilic bacteria represent a special group of prokaryotes that prefer to grow in very alkaline environments but show little or no growth at near-neutral pH values. Recent studies have revealed a remarkable diversity of alkaliphiles with representatives in most of the major taxonomic groups (Jones et al. 1998; Jones and Grant 1999). Much of the earlier research focused on gram-positive alkaliphiles assigned to the genus *Bacillus*. These strains are relatively easy to isolate from nonspecific environments, and many of them produce extracellular hydrolytic enzymes active at high pH, making them attractive for biotechnological studies of industrial enzymes (Horikoshi 1991, 1998).

Gram-negative alkaliphilic bacteria with the exception of anaerobic phototrophs have been poorly investigated. Only recently specific studies on the microbial composition of the highly alkaline soda lakes revealed the presence of a diverse community of gram-negative chemoorganotrophic alkaliphilic bacteria (Duckworth et al. 1996; Jones et al. 1994, 1998). Almost nothing is known about alkaliphilic aerobic chemolithoautotrophic bacteria that perform the oxidation of the final products of anaerobic destruction of organic matter, such as methane, hydrogen, ammonia, and sulfide. That such anaerobic processes are very active in most of the shallow and eutrophic soda lakes is evident from the few microbiological field studies in these environment (Isachenko 1951; Abdel Malek and Rizk 1963; Imhoff et al. 1979; Zavarzin 1993). A recent search for methane-oxidizing bacteria in the soda lakes of Tuva (Central Asia) resulted in the isolation of a halotolerant and moderately alkaliphilic methanotrophic bacterium described as "*Methylobacter alcaliphilus*" (Khmelina et al. 1996, 1997a,b).

Studies aimed at an investigation of aerobic chemolithotrophic bacteria in alkaline environments have also

demonstrated the presence of a diverse population of alkaliphilic, obligately autotrophic, and heterotrophic sulfur-oxidizing bacteria in the Siberian and African soda lakes (Sorokin et al. 1996a,b).

This article describes properties of a newly isolated, obligately methylotrophic, methane-oxidizing bacterium from Kenyan soda lakes. The isolate differed from the known neutrophilic methanotrophic species in its ability to grow and oxidize methane and ammonia under highly alkaline conditions. This knowledge further extends the taxonomic and physiological groups of prokaryotes found to be present in these environments, as well as providing added insight into the recycling of nutrients and minerals.

Material and methods

Samples

A composite sample of surface sediments from the Kenyan soda lakes Bogoria, Magadi, Elmenteita, Nakuru, and Little Naivasha (pH 10–10.5) was used to enrich for alkaliphilic methane-oxidizing bacteria. The samples were obtained in December 1996, transported to the laboratory, and kept at 4°C before use.

Strains

For comparison, the biomass of the alkaliphilic methanotroph "*Methylobacter alcaliphilus*" 20Z and the type strains of neutrophilic methanotrophs from group I (see Table 5) were kindly provided by Y. Trotsenko from the special collection of the Institute of Biochemistry and Physiology of Microorganisms (Puschino, Russia).

Isolation procedure

The enrichment was performed in 100-ml bottles with butyl rubber stoppers and 20 ml mineral base medium at a starting pH of 10.1. The mineral base includes Na_2C0_3, 23 g/l; $NaHCO_3$, 7 g/l; NaCl, 5 g/l; K_2HPO_4, 0.5 g/l; KNO_3, 5 mM; $MgSO_4 \cdot 7H_2O$, 0.5 mM; and trace elements, 2 ml/l (Pfennig and Lippert 1966). The headspace comprised an air–CH_4 mixture at a 50:50 ratio. Enrichment cultures were incubated at 30°C with gentle shaking. After several successful passages with 1:100 dilution, a serial dilution was made, and a culture from the last positive dilution was used for the isolation of a pure culture from the single colonies that appeared on mineral agar at pH 10 during prolonged incubation of the plates under an air–CH_4 atmosphere. Single colonies were transferred into a mineral liquid medium and the cultures showing growth with methane were selected. The purity of the selected clones was checked by repeated passages onto a solid medium and by the absence of growth on rich organic media.

Batch cultivation

Routine batch cultivation was performed at pH 10 using the same mineral base as indicated above. Media with different pH were prepared on the basis of HEPES-potassium phosphate buffer (pH 7–8) supplemented with 0.6 M NaCl or on the basis of sodium carbonate-sodium bicarbonate (pH 9–10.5) with 0.6 M total Na^+. Buffers were supplemented with KNO_3, K_2HPO_4 (soda buffers), and trace elements after being filter sterilized. Batch cultivation in small volumes (to 100 ml) was performed in closed jars filled with an air–CH_4 (50:50) mixture. Large-scale cultivation was performed in 20-l bottles with 5 l liquid culture under the same gas mixture. All growth experiments were performed at 30°C.

Experiments with washed cells

Kinetic parameters of substrate oxidation and the influence of pH on cell activity was measured using cell suspensions obtained from batch cultures grown with methane at pH 10. The activity of the oxidation of C_1 substrates and nitrogen compounds was studied by measuring the oxygen consumption with a Clark-type oxygen electrode in a 5-ml thermostated cell (Yellow Spring, Co., OH, USA). The rate of ammonia oxidation was also measured directly by following the nitrite production in cell suspensions under different conditions. Buffers for the measurements were the same as used for the batch cultivation, with 0.6 M total Na^+, except that potassium phosphate and potassium nitrate were replaced by 50 mM KCl.

Enzyme assays

The activity of the key enzymes of C-1 utilization was measured in the cell-free extract obtained by ultrasonic treatment of the cells grown with methane at pH 10. The assay was performed at pH 7.5 in 0.05 M potassium phosphate buffer. The activity of hexulose-6-phosphate synthase (RuMP pathway) was measured according to Kato (1990) and the activity of hydroxypyruvate reductase (serine pathway) according to Goodwin (1990).

Genetic analysis

Total DNA extraction, purification, and subsequent analysis of DNA base composition and DNA–DNA hybridization were performed according to Marmur (1961) and De Ley et al. (1970).

16*S* rDNA sequence and phylogenetic analysis

The 16*S* rDNA sequence analysis was performed and the results were interpreted by the BCCM™/LMG identification service (University of Gent, Belgium). Genomic DNA was extracted and purified according to the protocol of Pitcher et al. (1989). The 16*S* rRNA gene was amplified by PCR using primers 16F27 and 16R1522 (positions 8–27 and

1541–1522, *E. coli* numbering system). Products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The almost complete sequence (1486 bp) was determined using a DNA Sequencer (model 377; Perkin Elmer, Foster City, CA, USA) and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (with AmpliTaq DNA Polymerase, Fs).

For a phylogenetic analysis, the sequence was aligned by using the sequence library of EMBL. A resulting tree was constructed using the neighbor-joining method.

Chemical analysis

Cell protein was determined by the Lowry method. Nitrite, hydroxylamine, and formaldehyde concentrations were measured colorimetrically according to Gries-Romijn van Eck (1966), Frear and Burell (1955), and Nash (1953), respectively.

Results

Enrichment of alkaliphilic methane-oxidizing bacteria

Visible growth with methane at pH 10 was observed only after 1 month incubation of a culture inoculated with sediment sample. Soil samples were not active. The positive culture was subcultured several times with 1:100 dilution until stable growth, entirely dependent on methane, was obtained. During the first stage of enrichment, it was found that the culture grew faster in closed jars filled with a 50:50 methane–air mixture than in closed bottles. Serial dilutions of the stable, enriched population gave positive results up to the 10^{-8} dilution. This dilution was plated onto mineral agar at pH 10. Plates were incubated under methane–air atmosphere, and after 10 days three types of colonies appeared. Each type was subcultured on liquid mineral medium. Only cultures inoculated with the most refractile, reddish, and compact colonies gave vigorous growth with methane. These cultures did not grow on organic media. Repeated streaking of these cultures onto the mineral plates and microscopic examination established the purity of the isolated bacterium. One of the strains was designated as AMO 1 and selected for further investigation.

Morphology

Strain AMO 1 had large (1–1.5 × 2–3 μm), motile, ovoid, sometimes coccoid cells (Fig. 1a). Cells usually possessed only one polar flagellum, but a small fraction may have up to three peritrichous flagella (Fig. 1b). Older cultures formed flocks containing slimy material with embedded coccoid cells with degraded cytoplasm. Refractile cells, filled with polyglucose-like storage material, were also observed in older cultures (Fig. 1c). Such cultures rapidly lost methane-oxidizing activity and viability. Thin sections revealed the presence of intracellular bundles of membrane disks typical for type I methanotrophs (Fig. 1d), and the presence of an additional subunit layer loosely associated with the cell wall surface (Fig. 1e), which was arranged in a hexagonal form as visualized after partial lysis of the cells in distilled water (Fig. 1f).

Growth in batch culture

General

AMO 1 was routinely cultivated on liquid medium at pH 10 (as described for enrichment) under methane–air 1:1. Active growth was always preceded by a lag phase of about 15–20 h. The partial pressure of methane could be decreased as much as 20% without influence on the final yield, but an increase in the oxygen concentration to 20% (instead of 10%) resulted in growth inhibition. Growth was stimulated by the addition of Cu^{2+}, to 15 μM. The maximum growth yield and the maximum specific growth rate achieved in batch culture at pH 10 in the presence of 10 μM Cu^{2+} were about 450 mg cell protein l^{-1} and 0.12 h^{-1}, respectively. These values are within the range known for the neutrophilic methanotrophs (Leak and Dalton 1986; Graham et al. 1993). AMO 1 can also be grown with low concentrations of methanol (2–5 mM), but the bacterium was gradually inactivated after three passages. One of the possible reasons for growth inhibition might be the excessive formaldehyde production (positive qualitative test with Nash reagent) resulting from the low formaldehyde-oxidizing activity (see following). Formate and formaldehyde did not support growth.

Nitrate was routinely used as a nitrogen source during cultivation of AMO 1. It can also be grown with nitrite (5 mM), but it did not utilize urea or organic nitrogen (amino acids). Growth was possible with low concentrations of ammonia at pH 10 as well, but high concentrations were inhibitory. The bacterium grew well when 2 mM of NH_4Cl was supplied to the liquid medium with a liquid to gas ratio of 1:5. Addition of 5 mM of NH_4Cl under the same conditions resulted in a lag phase of 3 days, but an adapted culture grew in presence of 5 mM of NH_4Cl without a lag phase. Nevertheless, the growth yield in cultures grown with ammonia was at least 1.5 times less than with nitrate alone, even if nitrate was added to ammonium-grown cultures. Moreover, at stationary phase in presence of ammonia, the culture aggregated in the same way as it did while growing on methanol, this being an indication of cell damage. AMO 1 was not able to grow without adding a bound nitrogen source, implying the absence of the ability to fix N_2.

The bacterium was able to grow with methane in the presence of up to 0.5 M NaCl in addition to the basic medium containing 0.6 M Na^+ as sodium carbonate/hydrogen carbonate. Addition of 1 M NaCl to a carbonate base inhibited growth completely.

Influence of pH on growth with methane in batch culture

Strain AMO 1 grew well at a starting pH between 9 and 10 (Table 1). Growth at pH lower than 9 and higher than 10

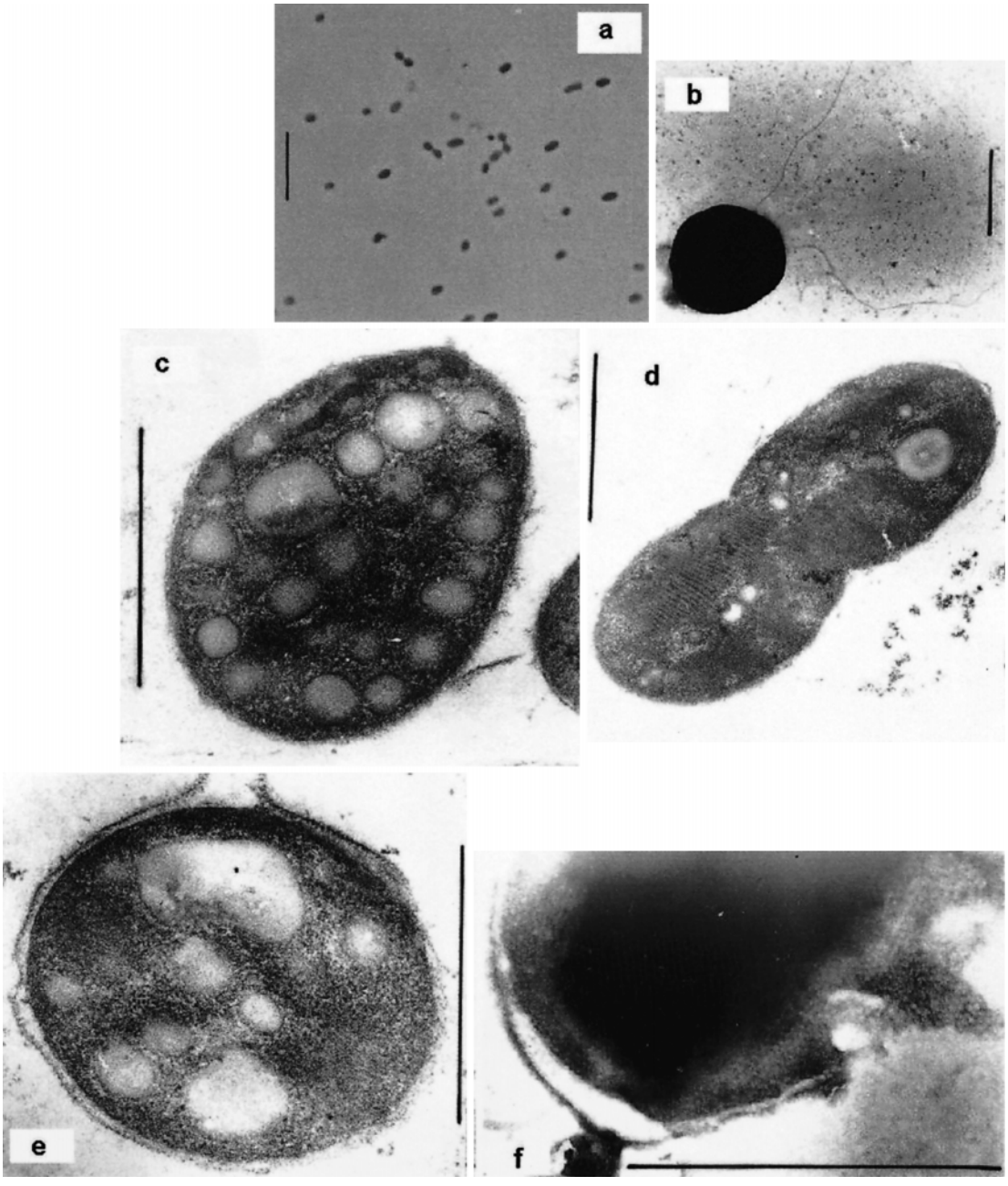


Fig. 1a–f. Morphology of the alkaliphilic methanotroph strain AMO 1 grown with methane at pH 10. **a** Phase-contrast photomicrograph. *Bar* 10 μm. **b–f** Electron photomicrographs. *Bar* 1 μm. **b,f** Total preparations. **c** Refractile cell from an old culture, thin section. **d** Thin section with intracellular membranes. **e** Thin section with a *S*-layer. **f** Partially lysed cell demonstrates hexagonal arrangement of the subunits in the *S*-layer. *ICPM*, intracytoplasmic membranes; *PG*, polyglucose granules

Table 1. The influence of the pH on the growth of the alkaliphilic methanotrophic bacterium strain AMO 1 with methane (CH_4/air 1:1, Cu^{2+} 10 μM)

Culture conditions		Growth		
Buffer	Initial pH	Growth period (h)	Biomass (mg prot/l)	Final pH
HEPES + NaCl, 0.6 M	7.3	90	40	7.3
	8.3	90	25	8.2
Sodium carbonate, 0.6 M Na^+	9.2	90	108	9.5
	10.05	40	360	9.9
	10.5	90 (aggr)[a]	25	10.2

[a] Cell aggregation and damage

was very slow and unstable so that it was not possible to obtain reproducible results. At a starting pH value of 10.5, slow growth began after 2 days with further aggregation of the biomass despite the spontaneous decrease of pH to 10.2.

Activity of the resting cells grown with methane at pH 10

The kinetic constants and pH profile of the activity of resting cells of AMO 1 were studied by measuring the rates of substrate-dependent oxygen consumption with an oxygen electrode (Fig. 2). The rates of CH_4 and methanol oxidation were almost equal but were four to five times higher than that of formaldehyde oxidation. Formate did not stimulate oxygen consumption. Growth under Cu^{2+} limitation resulted in a substantial decrease of CH_4 oxidation. The affinity constants for methane, methanol, and formaldehyde oxidation, measured at pH 10.0, were 16 μM, 0.6 mM, and 1.3 mM, respectively. The respiratory activity of resting cells of AMO 1 was dependent on the presence of K^+ ion; 50 mM K^+ was sufficient for maximum activity. Addition of 1 M NaCl to a soda buffer, initially containing 0.6 M Na^+, caused a 75% inhibition of CH_4 oxidation at pH 10.

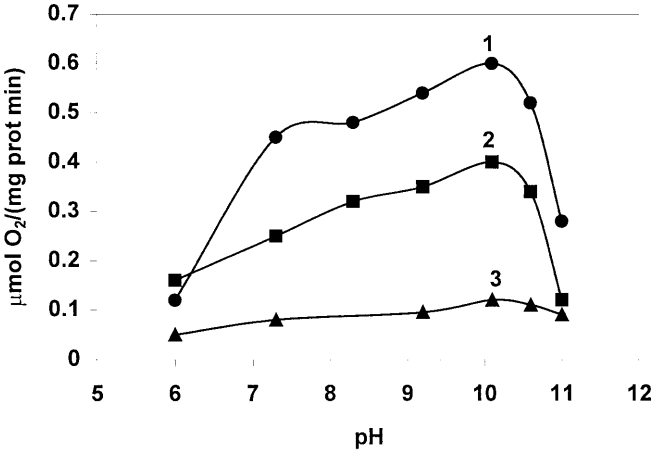


Fig. 2. Respiratory activity of resting cells of alkaliphilic methanotroph strain AMO 1 grown with methane at pH 10 in the presence of 10 μM Cu^{2+}: *1*, with methane; *2*, with methanol; *3*, with formaldehyde. Buffers: pH 6–8, HEPES + 0.6 M NaCl + 0.05 M KCl; pH 9–11, sodium carbonate-sodium bicarbonate (0.6 M total Na^+) + 0.05 M KCl; biomass, 0.15 mg prot ml^{-1}

Key enzymes of carbon assimilation

The activity of the key enzymes of ribulose monophosphate and serine pathways of carbon assimilation was measured in the cell-free extract prepared from the cells of AMO 1 grown at pH 10 with methane. Activity of hydroxypyruvate reductase, a key enzyme of serine pathway, was not detectable. The activity of hexulose-6-phosphate synthase of the ribulose monophosphate route, assayed by direct measurement of formaldehyde consumption, was relatively high, 750–850 nmol · mg $prot^{-1}$ · min^{-1}. This result is a strong indication that this alkaliphilic isolate belongs to the type I methanotrophs.

Ammonia oxidation by strain AMO 1

Batch culture

Both methane- and methanol-grown cultures of strain AMO 1 produced some nitrite from ammonia at pH 10. No nitrite was produced in the absence of ammonia (e.g., by nitrate reduction). Therefore, the alkaliphilic methanotroph strain AMO 1 can be regarded as the first known example of a pure bacterial culture able to oxidize ammonia at such high pH. The amount of nitrite produced was much less than in cultures of autotrophic nitrifiers but within the range usually found in cultures of neutrophilic methanotrophs grown with ammonium and methane or methanol (Bedard and Knowles 1989).

The maximum nitrite concentration in cultures grown with 5 mM of methanol was 0.22 mM, but, as mentioned earlier, such cultures were gradually inactivated. In contrast, AMO 1 grew well in presence of low concentrations of NH_3 with methane and nitrate (pH 10). The amount of nitrite produced in such cultures depended on the initial methane concentration in the gas phase (Table 2). The maximum nitrite formation was observed with 10% CH_4 in the gas phase (up to 0.62 mM). Most of the nitrite was produced in the stationary phase during aggregation of the cells when a substantial portion of the methane had already been consumed. Therefore, the actual optimal methane concentration for nitrite production should be less than 10% in the gas phase. Higher concentrations of CH_4 and the absence of nitrate as nitrogen source inhibited nitrite formation.

Table 2. Nitrite production from ammonia by the alkaliphilic methanotrophic bacterium strain AMO 1 grown with CH_4 at pH 10

CH_4, % in gas phase	Growth and nitrite production						
	26 h		46 h		68 h		Final pH
	Biomass OD_{600}	NO_2^-, μM	Biomass, OD_{600}	NO_2^-, μM	Biomass, OD_{600}	NO_2^-, μM	
5	0.11	30	0.34	86	aggr.	130	9.77
10	0.34	52	0.90	270	aggr.	620	9.63
20	0.37	17	0.75 aggr.	85	aggr.	95	9.7
50	0.38	10	0.90 aggr.	22	aggr.	33	9.7

O_2, 10% in gas phase; NH_4^+, 5 mM; NO_3^-, 5 mM

Washed cells

Resting cells of AMO 1, even grown without ammonia, were able to produce nitrite from ammonia and exhibited NH_3-dependent oxygen consumption. The maximum rate of ammonia-stimulated oxygen consumption by the cell suspensions was observed at pH 10–10.6 (Fig. 3). The rate was higher than the known values for neutrophilic methane-oxidizing bacteria. The affinity constant for ammonia at pH 10 was about 1 mM. At high concentration, ammonia caused inhibition of the oxygen consumption. The toxicity increased with increasing pH. The K_{i50} value decreased from 12 to 2 mM when increasing pH from 10.0 to 11.0. A test of the ability of strain AMO 1 to oxidize hydroxylamine was complicated by the chemical instability of this compound at high pH values. Nevertheless, it was found that AMO 1 can oxidize NH_2OH with rates of 50 and 35 nmol $O_2 \cdot mg\,prot^{-1} \cdot min^{-1}$ at pH 7.3 and 10.0, respectively.

The high pH optimum for ammonia oxidation in this alkaliphilic methanotroph was confirmed by direct measurement of nitrite production by the same washed cells in the same buffers (see Fig. 3). However, the actual specific nitrite formation rate was three to four times lower than could be expected on the basis of the ammonium-dependent oxygen consumption, assuming 1 NO_2^- formed per 1.5 [O] consumed. Several explanations are possible. The most obvious is that ammonia might not only act as a source of electrons, but also might stimulate the oxidation of the internal storage material (polyglucose). Methanol and methane at low concentrations substantially stimulated nitrite production from ammonia by washed cells of AMO 1 at pH 10 (Fig. 4), while formaldehyde and formate inhibited nitrite production at concentrations of 1–2 mM.

Oxidation of organic sulfur

The ability to oxidize a wide range of various organic compounds via methane monooxygenase is a well-known property of methanotrophic bacteria (Sullivan 1998). It was of particular interest to check the potential of the alkaliphilic isolate AMO 1 to oxidize organic sulfur because our previously isolated sulfur-oxidizing alkaliphiles were active only with inorganic sulfur species. Washed cells of strain AMO 1 grown with methane were indeed capable of active CS_2 oxidation at pH 10.0 (Table 3). The activity of CS_2 and methane-dependent oxygen consumption were both maximal in cells obtained from exponentially growing culture. Very low but detectable activity of oxygen consumption was also observed with DMS (dimethyl sulfide) and DMDS (dimethyl disulfide), but not with methanethiol or DMSO (dimethylsulfoxide). The product of CS_2 oxidation by strain AMO 1 was qualitatively identified as a polysulfide (yellow color with light absorption between 380 and 440 nm, sulfur plus sulfide formation after acidification). The addition of a small amount of washed cells of the alkaliphilic autotrophic sulfur-oxidizing bacterium strain AL 18 (previously isolated

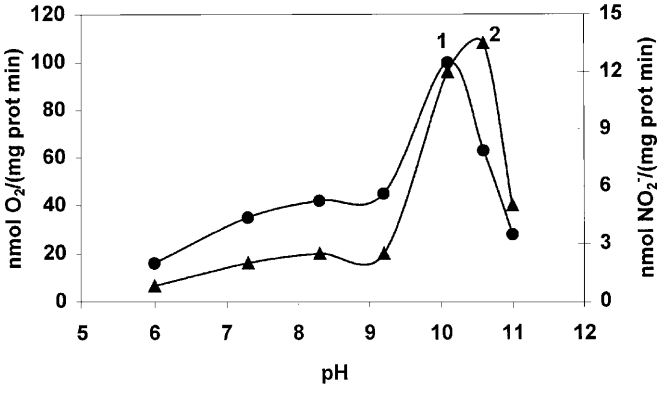
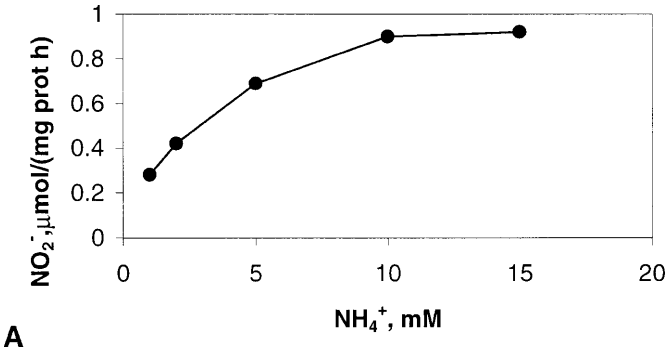
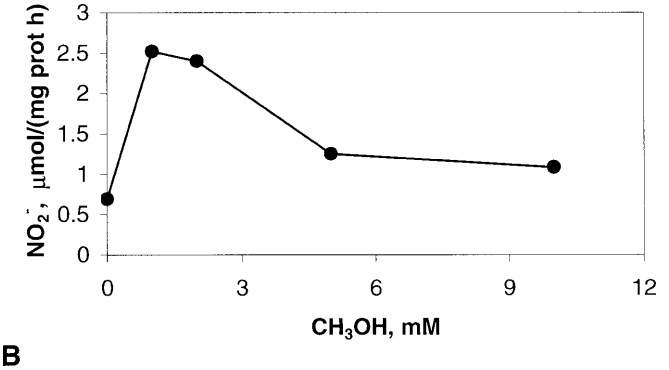


Fig. 3. Influence of pH on the ammonia-oxidizing activity of washed cells of strain AMO 1 (all conditions, as indicated in Fig. 2): *1*, ammonia-dependent oxygen consumption; *2*, nitrite production





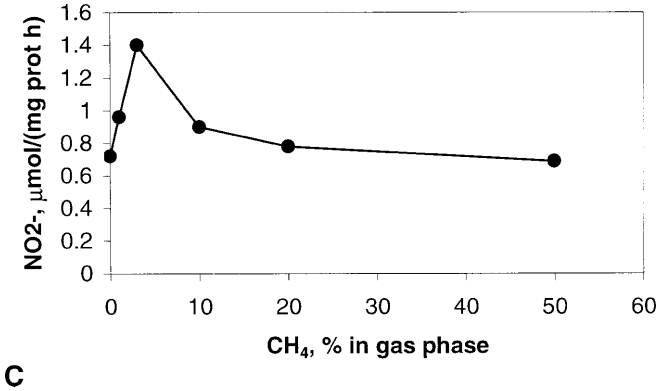


Fig. 4A–C. Nitrite production from ammonia at pH 10.1 by washed cells of strain AMO 1 (all conditions, as indicated in Fig. 2). **A** NH_4^+ without additional electron donors; the influence of ammonia concentration. **B** In the presence of methanol (NH_4^+, 5 mM). **C** In the presence of methane (NH_4^+, 10 mM)

Table 3. Oxidation of organic sulfur compounds by resting cells of the alkaliphilic methanotrophic strain AMO 1, grown with methane at pH 10

Cells	Substrate	Respiration rate, nmol O_2/(mg prot min)	Detected sulfur products
AMO 1 (stationary phase)	CH_4	680	
	CS_2	100	Polysulfide
	S_6^{2-}	0	
AL 18	CS_2	0	
	S_6^{2-}	2700	S_o
AMO 1 + AL 18	CS_2	170	S_o
AMO 1, exponential phase	CH_4	1050	
	DMS	20	
	DMDS	16	
	DMSO	4	
	CS_2	290	Polysulfide
Same + AL 18	CS_2	460	S_o

Substrate-dependent oxygen consumption was measured in a soda buffer (0.6 M total Na^+, pH 10.05). Cells of strain AMO 1 were added to a final concentration 0.2–0.25 mg prot/ml. In some experiments with CS_2, washed cells of an alkaliphilic sulfur-oxidizing bacterium strain AL 18 (grown with thiosulfate at pH 10) were added to a final concentration 0.05 mg prot/ml together with the AMO 1 cells. All organic sulfur compounds were supplied as acetone solutions to final concentration of 100 μM (acetone itself had no influence on respiration of strain AMO 1). Methane was added as a saturated water solution, 0.5–4.5 ml soda buffer. Endogenous respiration rates were substracted from the substrate-dependent rates. The chemical rate of oxidation (buffer without cells) of the substrates was insignificant

Table 4. Comparision of the properties of the alkaliphilic methanotrophic isolate AMO 1, the marine methanotroph *Methylomicrobium pelagicum*[a] and "*Methylobacter alcaliphilus*" 20 Z[b]

Property	AMO 1	*Methylomicrobium pelagicum*	*"Methylobacter alcaliphilus"* 20 Z
Cell morphology:			
Cell shape	Ovoid rods to cocci	Coccobacilli	Rods
Flagella	1–3	1	1
S-layer in cell wall	+ (globular)	−	+ (cup-shaped)
Major inclusions	polyglucose	Polyphosphate	
Carbon and energy source	Methane, methanol	Methane, methanol	Methane, methanol
Nitrogen source	Ammonia, nitrate, nitrite	Ammonia, nitrate, aspartate	Ammonia, nitrate
Formaldehyde-oxidizing activity	Low	Low	
Growth with methane at:			
pH 5.5	−	+	−
pH 9–10	+	−	+
Total Na^+	To 1.1 M	To 0.8 M	To 1.5 M
GC mol% in DNA (Tm)	50.9	49	47.6
Source	Soda lake sediments (Kenya)	Seawater	Soda lake (SE Siberia)

[a] From Sieburth et al. (1987); Bowman et al. (1993, 1995)
[b] From Khemelina et al. (1997a,b)

from a Siberian soda lake; unpublished data) to a suspension of the alkaliphilic methanotroph increased the CS_2-dependent oxygen consumption rate (Table 3). Strain AL 18 was unable to oxidize CS_2 but actively oxidized sulfane atoms of polysulfide to produce elemental sulfur and sulfate. Indeed, a mixed-cell suspension of AMO 1 and AL 18 produced elemental sulfur instead of polysulfide, and the overall CS_2-dependent respiration rates increased, as compared to pure AMO 1 cells.

Genetic analysis

DNA analysis

The alkaliphilic isolate AMO 1, in general, resembled the previously described alkaliphilic methanotroph "*Methylobacter alcaliphilus*" 20 Z (Khmelina et al. 1997a,b), except for some differences in the G + C content of the DNA, ultrastructure, and pH response (Table 4). Based on the formal criteria, both alkaliphilic strains AMO 1 and 20 Z can be classified as *Methylobacter* species. However, the results of DNA–DNA hybridization revealed that the alkaliphilic methanotrophs AMO 1 and 20 Z are not closely related and that they are equally distant from the neutrophilic representatives of the genera *Methylobacter* and *Methylomicrobium* (Table 5).

Phylogenetic analysis

According to the results of 16*S* rDNA gene sequence, the alkaliphilic isolate AMO 1 belongs to the gamma-*Proteobacteria* with highest similarity level of 96%–

Table 5. DNA homology between the alkaliphilic and neutrophilic group I methanotrophs

Strain	GC mol%	DNA–DNA homology, %			
		20Z	AMO 1	*Methylobacter vinelandii*	*Methylobacter bovis*
"*Methylobacter alcaliphilus*" 20Z	47.9	100			
AMO 1	50.9	21	100		
Methylobacter vinelandii 30	52.3	28	23	100	
Methylobacter bovis 98	52.1	26	26	81	100
Methylomicrobium album 16	52.7	21	24	16	12

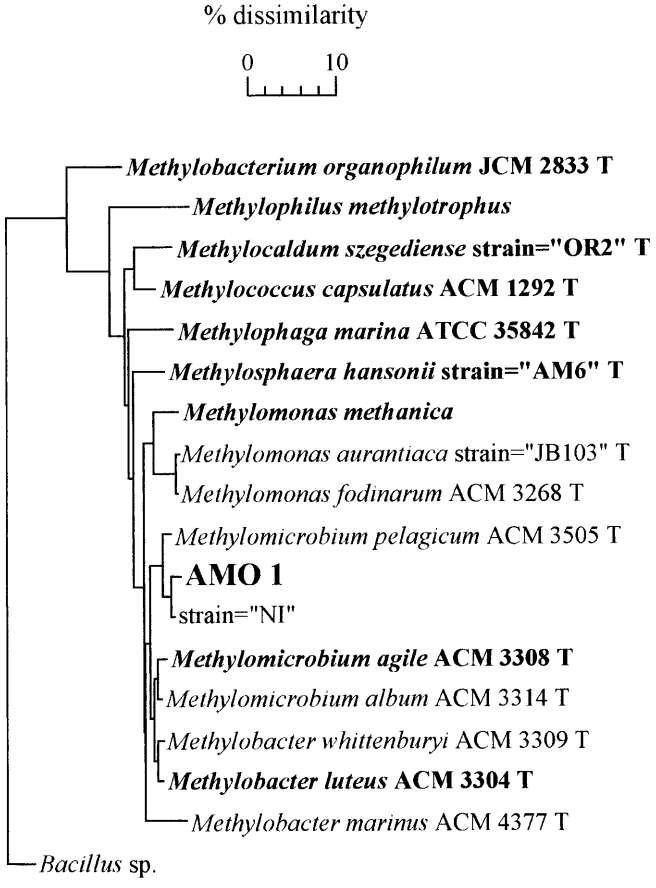


Fig. 5. Phylogenetic tree showing the relationship between the alkaliphilic methanotrophic isolate strain AMO 1 and neutrophilic methanotrophic bacteria of the gamma-*Proteobacteria*; type species are shown in ***bold***

98.5% to different strains of the neutrophilic marine methanotroph *Methylomicrobium pelagicum* (formerly *Methylomonas pelagica*) (Sieburth et al. 1987). The highest similarity (98.5%) was found with a recently described DMS-oxidizing *M. pelagicum*-like neutrophilic marine methanotroph strain NI (Fuse et al. 1998). "*Methylobacter alcaliphilus*" 20Z is also a member of this cluster with a sequence similarity of about 97% with strain AMO 1 (T. Tourova, personal communication). The sequence similarity with the other methanotrophic species in the gamma-*Proteobacteria* from the genera *Methylobacter* and *Methylomonas* was in the range of 93%–95% (Fig. 5). The 16*S* rDNA sequence data of strain AMO 1 has been deposited in EMBL and GenBank databases with the accession number AJ132384.

Discussion

Methane oxidation

On the basis of physiological experiments, strain AMO 1 isolated from alkaline environments can be considered as a second known representative of the alkaliphilic methane-oxidizing bacteria. Most of the previously investigated obligate methanotrophs were isolated from neutral environments, and the upper pH limit for their growth and oxidizing activity does not exceed pH 9.0 (Dalton 1977; Bowman et al. 1993; Hanson and Hanson 1996). Recently, new types of methanotrophs were found in extreme environments: the psychrophilic community in the tundra (Omel'chenko et al. 1992), acidophilic oligotrophic representatives in peatlands (Dedysh et al. 1998), and halotolerant moderate alkaliphiles in Siberian soda lakes (Khmelina et al. 1996, 1997a,b). The former and the latter are represented by *Methylobacter*-like organisms, demonstrating the high potential of these methanotrophic bacteria for adaptation to various environments.

Our data demonstrate the presence of methane-oxidizing bacteria in extremely alkaline and saline Kenyan soda lakes. Among other extreme environments, hypersaline waters and hyperthermal habitats seem to lack an active aerobic methane-oxidizing community (Slobodkin and Zavarzin 1992; Conrad et al. 1993). One of the reasons may be the limited gas solubility at high salt concentration and at high temperature. In this connection, it is interesting to note that the alkaliphilic methane-oxidizing isolate described here was relatively salt sensitive as compared to the sulfur-oxidizing autotrophic alkaliphilic bacteria, isolated from the same Kenyan soda lakes. While strain AMO 1 did not tolerate more than 1 M of total Na^+, most of the sulfur-oxidizing alkaliphiles could be grown up to 1.5 M of total Na^+ and some of them even up to 4 M (unpublished data).

The growth experiments at different pH clearly demonstrated that strain AMO 1 prefers to grow at pH values between 9 and 10, implying that the bacterium belongs to the alkaliphiles. However, very slow growth occurred also at neutral pH. Its alkaliphilic nature is also supported by the pattern of the pH profile of its respiratory activity. It was

not possible to find the upper pH limit for the growth of this isolate because even the strong buffering capacity of the soda-based medium was not sufficient to keep the pH higher than 10.1 in methane-oxidizing batch cultures. Therefore, the true values of the pH profile for growth can be obtained only by using pH-controlled cultivation, preferably in continuous culture. The latter approach may also be useful to investigate the potential for growth under methanol limitation.

The maximum rate of methane oxidation by strain AMO 1 fell into the middle of the scale known for the neutrophilic methylotrophs, 0.2–2 $\mu mol\, O_2 \cdot mg\, prot^{-1} \cdot min^{-1}$ (Bedard and Knowles 1989). The main difference was in the pattern of the pH profiles of the CH_4-monooxygenase and methanol- and formaldehyde-dependent oxidizing systems. In the alkaliphilic strain AMO 1, all these activities continued almost equally well over a broad pH range between 7 and 10.5, being 50% from the maximum at pH 11.0, whereas CH_4-dependent respiration in neutrophilic species was totally inactivated at pH 9.0 and higher (Dalton 1977; Hanson and Hanson 1996).

Ammonia oxidation

Strain AMO 1 was capable of ammonia oxidation at extremely high pH, although not so actively as autotrophic nitrifiers at neutral conditions. This property is common among neutrophilic methane-oxidizing bacteria (Hanson and Hanson 1996) and is associated with the low specificity of methane monooxygenase (Dalton 1977; Bedard and Knowles 1989). In contrast to its methane-oxidizing activity, ammonia oxidation by strain AMO 1 had a sharp maximum at very high pH values (10–10.5). In the neutrophilic bacterium *Methylococcus capsulatus*, the pH optimum for ammonia oxidation by washed cells was also higher than for methane, but both substrates were not oxidized at all above pH 9.0 (Dalton 1977). The stimulation of nitrite production by low concentrations of methane and methanol, as observed with strain AMO 1, was also common among the neutrophilic methanotrophs (O'Neil and Wilkinson 1977).

In view of the fact that so far it has not been possible to isolate alkaliphilic autotrophic ammonia oxidizing bacteria from the soda lakes, the observed nitrifying potential of strain AMO 1 at pH values 10 and even higher is an interesting phenomenon. At least it is clear that ammonia oxidation itself is possible at very high pH, and that the main limitations are probably ammonia toxicity and availability under such conditions. Whether the methanotrophs could be involved in ammonia oxidation in alkaline natural environments remains to be investigated. Direct measurements of CH_4 oxidation rates and indirect measurements of nitrification rates in the alkaline meromictic Mono Lake (Sierra Nevada, CA, USA; pH about 9.7) demonstrated that the methanotrophic population there was much less active than the NH_3-oxidizing population and that the methanotrophs are not involved in the nitrification process. Moreover, the methanotrophic activity was already inhibited at 0.3 mM of NH_3 at high pH (Joye et al. 1999). The situation might be different, however, in the highly productive, shallow Kenyan or Siberian soda lakes.

Organic sulfur oxidation

Active CS_2 oxidation at high pH by the alkaliphilic isolate AMO 1 represents an interesting addition to many other known cooxidation reactions carried out by methanotrophic bacteria because of the low specificity of their soluble or membrane-bound MMO. CS_2 is a well-known inhibitor of ammonium monooxygenase (Hyman et al. 1990). Moreover, the possibility of CS_2 oxidation (as well as DMS) by nongrowing *Nitrosomonas europea* cells has been reported (Juliette et al. 1993), although the product of oxidation was not identified. DMS was oxidized to DMSO. Assuming a substrate specificity resemblance between AMO and MMO, one might suggest that CS_2 may also be a substrate for MMO, although there is no such evidence in the literature concerning the neutrophilic methanotrophs. However, the recent discovery of the ability to convert DMS to DMSO in a new marine *M. pelagicum*-like methanotroph (Fuse et al. 1998) suggests that MMO might also be responsible for oxidation of the sulfur atom in organic compounds.

Overall, the new alkaliphilic methanotrophic bacterium has potential for participation in three different oxidative cycles (C, N, and S) and might represent an important biogeochemical agent in the soda lake environment.

Taxonomic status

Phylogenetically, the alkaliphilic isolate AMO 1 was most closely related to the marine methanotroph *Methylomicrobium pelagicum* described by Sieburth et al. (1987). Although AMO 1 in general resembled *M. pelagicum*, there were substantial phenotypic differences (see Table 4), such as the number of flagella, the presence of a *S*-layer, and difference in GC mol%. Moreover, the main difference of AMO 1 from *M. pelagicum* is the alkaliphilic nature of the former. However, a direct genetic comparison with *M. pelagicum* seems now to be impossible as the strain is apparently no longer available. Accepting these differences, the alkaliphilic methanotroph AMO 1 could be described as a new species of the genus *Methylomicrobium*. However, the true status of this genus within the group I methanotrophs is uncertain because a recent comparision of the phylogenetic trees constructed by different authors and in different ways demonstrates that several species of *Methylomonas, Methylobacter*, and *Methylomicrobium* are intermixed (T. Tourova, personal communication). In particular, *Methylmicrobium (Mmb.) album* and *Mmb. agile* are clustered with *Methylobacter* species. On the other hand, several *Mmb. pelagicum* marine strains and salt-tolerant alkaliphilic isolates (AMO 1, 20Z, and 10Z) form a compact separate cluster. Although the alkaliphilic methanotroph strain AMO 1 superficially appears to represent a novel *Methylomicrobium* species, its true taxonomic status must await a more thorough investigation of the genus *Methylomicrobium*.

Description of *Methylomicrobium* sp., strain AMO 1

Gram-negative, large, ovoid rods, some times coccoid, 1–1.5 × 2–3 μm. Motile mostly by a single polar flagellum. A small fraction of the cells may possess up to three peritrichous flagella. Reproduces by binary fission. Cells have an additional external subunit layer in the cell wall. Intracytoplasmic membranes are typical for the type I methanotrophs. Refractive cells filled with polyglucose storage granules are formed in older cultures. Colonies on soda-mineral agar are slightly pink, 1–3 mm in diameter, regular, convex, with entire edge. Grows only with methane and methanol by using the RuMP pathway of carbon utilization. Nitrate and ammonia can serve as nitrogen sources. Mesophilic; maximum growth temperature at pH 10 is 40°C. Grows well in a narrow pH range between 9 and 10. Batch culture growth at pH lower than 9.0 and higher than 10.2 is very slow and unstable. Sodium ion is required for growth. Tolerates up to 1 M total sodium ion concentration during growth on methane at pH 10. Oxidizes ammonia to nitrite at pH values above 10.0. Methane-oxidizing and ammonia-oxidizing activity are maximal at pH near 10. The G + C content of the DNA is 50.9% ± 0.5% (T_m). Isolated from the surface sediments of Kenyan soda lakes. Strain AMO 1 is deposited with the bacterial collection of TU Delft (Delft, The Netherlands) under the number LMD 97.157 and with the National Collection of Industrial and Marine Bacteria (Aberdeen, UK) as NCIMB 13566.

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