

Nitrogen fixation by the verrucomicrobial methanotroph ‘*Methylacidiphilum fumariolicum*’ SolV

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The ability to utilize atmospheric nitrogen (N₂) as a sole nitrogen source is an important trait for prokaryotes. Knowledge of N₂ fixation by methanotrophs is needed to understand their role in nitrogen cycling in different environments. The verrucomicrobial methanotroph ‘*Methylacidiphilum fumariolicum*’ strain SolV was investigated for its ability to fix N₂.

Physiological studies were combined with nitrogenase activity measurements and phylogenetic analysis of the *nifDHK* genes, encoding the subunits of the nitrogenase. ‘*M. fumariolicum*’ SolV was able to fix N₂ at low oxygen (O₂) concentration (0.5%, v/v) in chemostat cultures. This low oxygen concentration was also required for an optimal nitrogenase activity [47.4 nmol ethylene h⁻¹ (mg cell dry weight)⁻¹]. Based on acetylene reduction assay and growth experiments, the nitrogenase of strain SolV seems to be extremely oxygen sensitive compared to most proteobacterial methanotrophs. The activity of the nitrogenase was not inhibited by ammonium concentrations up to 94 mM. This is believed to be the first report on the physiology of N₂ fixation within the phylum *Verrucomicrobia*.

INTRODUCTION

Biological N₂ fixation is essential to life. In this process nitrogen is taken from its relatively inert molecular form (N₂) in the atmosphere and converted into ammonium according to the equation $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$ (Dixon & Kahn 2004; Howard & Rees, 1996). The micro-organisms performing this process are known as diazotrophs and they provide about 60% of the annual nitrogen input into the biosphere (Newton, 2007). Diazotrophs are found in the domains Bacteria and Archaea and their lifestyle varies from free-living, loosely associated to symbiotic. The first methane-oxidizing bacteria, or methanotrophs, capable of N₂ fixation were isolated in 1964, referred to as *Pseudomonas methanitricans* (Davis *et al.*, 1964). Methanotrophs form an important sink for the greenhouse gas methane but the coupling between the methane and nitrogen cycles is poorly understood (Murrell & Jetten, 2009).

The microbial oxidation of methane may be coupled to the reduction of sulfate, nitrite or oxygen (Boetius *et al.*, 2000; Hanson & Hanson, 1996; Raghoebarsing *et al.*, 2006). Oxygen-consuming obligate aerobic methanotrophs are widespread in many natural environments (Conrad, 2009; Hanson & Hanson, 1996), where they feed on the methane

produced by methanogens in the anoxic zones of these ecosystems. Until recently, all methanotrophs could be phylogenetically placed into 13 genera, belonging to the *Gammaproteobacteria* (type I) and the *Alphaproteobacteria* (type II) (Hanson & Hanson, 1996). However, in 2007, three research groups independently described novel thermoacidophilic methanotrophs isolated from geothermal areas in Italy, New Zealand and Russia (Dunfield *et al.*, 2007; Islam *et al.*, 2008; Pol *et al.*, 2007). These methanotrophs represented a distinct phylogenetic lineage within the phylum *Verrucomicrobia* and they belong to a single genus for which the name ‘*Methylacidiphilum*’ was proposed (Op den Camp *et al.*, 2009). Environmental clone libraries show that there is a large biodiversity in verrucomicrobia and they are encountered in many ecosystems, such as soils, peat bogs, acid rock drainage and landfill leachate, often in relatively high numbers, but having an unknown physiology (Wagner & Horn, 2006). However, the verrucomicrobial methanotrophs were obtained in pure cultures and a complete genome sequence was published for ‘*Methylacidiphilum infernorum*’ strain V4 (Hou *et al.*, 2008) while a draft genome analysis was done for ‘*Methylacidiphilum fumariolicum*’ strain SolV (Pol *et al.*, 2007). As nitrogen is one of the compounds that limits bacterial growth in most ecosystems, the ability to utilize N₂ as a sole nitrogen source is an important trait. Knowledge of N₂ fixation by methanotrophs will help us

Two supplementary figures are available with the online version of this paper.

to understand their role in carbon and nitrogen cycling in different environments. Although it was first assumed that only type II and the type I moderately thermophilic *Methylococcus* strains were capable of N₂ fixation (Oakley & Murrell, 1988), later the presence of both *nifH* gene fragments and acetylene reduction activity was demonstrated in a variety of type I and type II strains (Auman *et al.*, 2001). Genetic and biochemical evidence was provided to show that N₂ fixation capabilities are broadly distributed among methanotrophs. Recently, it was also demonstrated that the deep-sea anaerobic methane-oxidizing Archaea fix N₂ and share the products with their sulfate-reducing bacterial symbionts (Dekas *et al.*, 2009).

Preliminary growth experiments and genome analyses indicated that also the '*Methylacidiphilum*' strains should be able to fix N₂ (Op den Camp *et al.*, 2009). The genomes of strain SolV and strain V4 show a complete set of necessary genes for N₂ fixation (Op den Camp *et al.*, 2009; Hou *et al.*, 2008). Most of these genes and their organization in putative operons resemble those of *Methylococcus capsulatus* Bath (Ward *et al.*, 2004), a gammaproteobacterial methanotroph that has been shown to fix N₂ (Oakley & Murrell, 1991). The aim of this study was to elucidate N₂ fixation by '*Methylacidiphilum fumariolicum*' SolV in more detail. Physiological studies were combined with nitrogenase activity measurements and analysis of the *nifH* gene encoding one of the subunits of the nitrogenase.

METHODS

Organism. '*Methylacidiphilum fumariolicum*' strain SolV used in this study was originally isolated from the volcanic region Campi Flegrei, near Naples, Italy (Pol *et al.*, 2007). The pH and temperature optima for growth were 2 and 55 °C, respectively.

Medium composition for growth. The medium used in this study to grow strain SolV contained: in g l⁻¹, MgCl₂·6H₂O, 0.08; CaHPO₄·2H₂O, 0.44; Na₂SO₄, 0.14; K₂SO₄, 0.35; (NH₄)₂SO₄, 0.26; plus 1 ml l⁻¹ trace element solution (Schönheit *et al.*, 1979) and 2% (v/v) autoclaved fangaia soil extract (liquid obtained from the Fangai mud pool at Pozzuoli in Italy), unless stated otherwise. The medium was adjusted to pH 2 with 1 M H₂SO₄ before autoclaving. To prevent precipitation, CaHPO₄·2H₂O was dissolved in fangaia soil extract and was sterilized separately and added to the bulk of the medium after autoclaving and cooling. The concentration of ammonium (NH₄⁺) in the fangaia soil extract may vary from 1 to 28 mM. Most of the experiments were performed with an extract containing 28 mM.

Gas and ammonium analyses. Methane (CH₄), oxygen (O₂), nitrogen (N₂) and carbon dioxide (CO₂) were analysed on an Agilent series 6890 gas chromatograph equipped with a Porapak Q and a Molecular Sieve column and a thermal conductivity detector as described before (Ettwig *et al.*, 2008). Ethylene (C₂H₄) and acetylene (C₂H₂) were analysed with a Varian star 3400 gas chromatograph using a flame-ionization detector and a Porapak N column (30 m × 0.250 mm) with helium (He) as carrier gas. The injection, detector and oven temperature were 125, 250 and 90 °C, respectively. The gas chromatographs were calibrated quantitatively for each gas by injecting dilutions of pure gases or mixtures. For all gas analyses 100 µl gas samples were injected with a glass syringe.

Ammonium concentrations were measured using the *o*-phthalaldehyde (OPA) method (Taylor *et al.* 1974).

Batch cultivation. The effect of O₂ concentration on N₂ fixation by strain SolV was tested in 1 l serum bottles, sealed with red butyl rubber stoppers. The batch incubations were performed in 50 ml nitrogen-free medium, without fangaia soil extract. The medium was inoculated with cells obtained from the early stationary phase and the inoculum size was always adjusted to achieve the same low initial optical density (OD₆₀₀), varying between 0.08 and 0.12. The gas composition in the headspace was adjusted to 13% and 8% (v/v) for CH₄ and CO₂, respectively. O₂ concentrations between 1 and 10% (v/v) were tested. The experiment was started by incubating the bottles at 50 °C with shaking at 200 r.p.m. During the experiment, gas samples were removed from the bottle headspace and analysed as described above.

Chemostat cultivation. The growth yield and stoichiometry of CH₄ conversion to CO₂ of strain SolV were determined using a chemostat, under N₂-fixing conditions. The chemostat, liquid volume 300 ml, was operated at 55 °C with stirring at 750 r.p.m. with a stirrer bar. The chemostat was supplied with medium at a flow rate of 5.1 ml h⁻¹, using a peristaltic pump. The cell-containing medium was removed automatically from the chemostat by a peristaltic pump when the liquid level reached the sensor in the reactor. Supply of CH₄ (0.72 ml min⁻¹), N₂ (0.46 ml min⁻¹) and CO₂ (2.1 ml min⁻¹) took place by mass flow controllers through a sterile filter and sparged into the medium just above the stirrer bar. An O₂ sensor in the liquid was coupled to an ADI1030 Biocontroller (Applikon) regulating the O₂ mass controller to achieve a O₂ saturation of 0.5%. After steady state was reached, CH₄ and O₂ consumption and CO₂ production were determined by measuring the ingoing and outgoing gas flows and the gas concentrations. The ingoing gas mixture now contained (all v/v) 14% CH₄, 19% O₂, 12% N₂ and 54% CO₂ at a flow rate of 3.8 ml h⁻¹. The outgoing gas passed through a sterile filter with a flow rate of 2.8 ml h⁻¹, and contained (all v/v) 3% CH₄, 2.8% O₂, 16% N₂ and 79% CO₂. To determine the dry weight concentration, triplicate 5 ml samples from the culture suspension were filtered through pre-weighed 0.45 µm filters and dried to constant weight in a vacuum oven at 70 °C.

Nitrogenase assay. Acetylene (C₂H₂) reduction has been shown to be a suitable assay for N₂ fixation in methanotrophs (Dalton & Whittenbury, 1976; Murrell & Dalton, 1983; Toukdarian & Lidstrom, 1984). The nitrogenase activity assay was performed in 120 ml serum bottles, sealed with grey butyl rubber stoppers. The black rubber stoppers proved to be inhibitory. For this assay 0.5–2 ml culture (1 mg dry weight) were used. For the acetylene reduction assay C₂H₂ and O₂ in the headspace were routinely set to be 2% and 0.5% (v/v), respectively. Because C₂H₂ is a potent inhibitor of the methane monooxygenase (Dalton & Whittenbury, 1976; De Bont & Mulder, 1976), 0.1% (v/v) methanol (CH₃OH) was provided to the cell suspension as an alternative electron donor. The experiment was started by incubating the bottles at 50 °C with shaking at 200 r.p.m. To measure the C₂H₄ production, the gas phase in the bottles was sampled at fixed time intervals and analysed by gas chromatography.

Phylogenetic analysis. Representative *nifH*, *nifD* and *nifK* gene sequences, encoding the functional proteins of the nitrogenase complex, were obtained from GenBank. The genes were also extracted from the preliminary genome data of strain SolV (GenBank accession no. GU299762). Conceptual translations into amino acids were performed and a concatenated set was created for alignment and phylogenetic analysis using MEGA 4 (Tamura *et al.*, 2007). The evolutionary history was inferred using the neighbour-joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred

from 500 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985).

RESULTS AND DISCUSSION

N₂ fixation in batch cultures

Although N₂ fixation by the recently discovered methanotrophic verrucomicrobia is claimed on the basis of preliminary data (Dunfield *et al.*, 2007) and the presence of their genetic potential (Op den Camp *et al.*, 2009) there are no reports that show N₂ fixation.

Batch incubations of *M. fumariolicum* SolV in nitrogen-free medium (and without fangaia soil extract) showed that this methanotroph was able to fix N₂. When *M. fumariolicum* cells from batch cultures in the late exponential phase (when ammonium was already depleted) were transferred to medium with N₂ as the sole source of nitrogen, and incubated at our standard oxygen concentrations (5–10%, v/v) some increase in OD₆₀₀ was observed but this increase did not last long. We subsequently used cells that had reached the stationary phase. Transfers of these cells (at 5–10%, v/v, O₂) never showed any growth within 10 weeks. However, when the initial oxygen concentration was reduced to 2% (v/v) an increase in OD₆₀₀ could be observed (Fig. 1a), indicating N₂ fixation. In batch cultures such low oxygen concentrations cannot be maintained because of rapid oxygen consumption. Even at low cell densities (OD₆₀₀ 0.1), repeated additions of oxygen were necessary and concentrations fluctuated accordingly (Fig. 1b). This variation was also described by Dedysh *et al.* (2004). Such incubations at around 2% (v/v) O₂ in the headspace resulted in linear

growth (Fig. 1a). After decreasing the oxygen concentration to 1.3% (v/v) and below, a short exponential growth phase was observed (Fig. 1b), but was experimentally difficult to reproduce. It was observed that in such bottles growth accelerated when oxygen concentrations dropped below 1% (v/v) and slowed down after adjusting the oxygen concentration to 1.5% (v/v).

In the above-described experiments, it took 13 days before the bacteria started to grow. When these (already) N₂-fixing cultures were transferred into fresh nitrogen-free medium, their lag phase was reduced to 1 day. The long adaptation is probably required for induction of the nitrogenase. As there is no free nitrogen available for this protein synthesis, the organism probably relies on the recycling of other proteins or the presence of some stored nitrogen. When the stationary-phase cultures were transferred to ammonium-containing medium, exponential growth started within 1 day, indicating that the long lag phase is not caused by inactivation of metabolism.

When a N₂-free argon (Ar) gas phase was used no growth was observed, further supporting the conclusion that an active N₂-fixing metabolism was present (Fig. 1a).

The requirement of low oxygen concentration for N₂ fixation has also been demonstrated for *Methylobacter luteus* (<2%, v/v), *Methylocystis* strain T-1 (<6%, v/v) and *Methylococcus capsulatus* Bath (<10%, v/v) by batch cultivation under N₂-fixing condition (Dedysh *et al.*, 2004; Murrell & Dalton, 1983; Takeda, 1988). In contrast some other known methanotrophs are able to fix N₂ at higher oxygen concentrations, e.g. *Methylosinus trichosporium* OB3b (15–17%, v/v) and *Methylocapsa acidiphila* B2^T (atmospheric oxygen concentration) (Dedysh *et al.*, 2002, 2004).

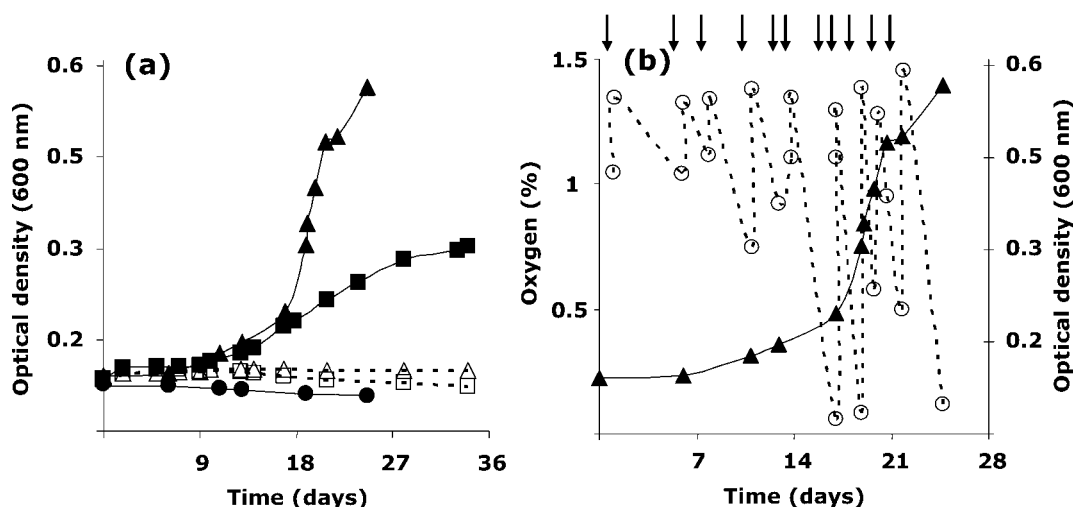


Fig. 1. (a) Batch cultivation of strain SolV in nitrogen-free medium with nitrogen gas as nitrogen source. Incubation with oxygen concentrations of between 0.5 and 1.5% (▲), between 1 and 2% (■), 5% (□) and 10% (△); and the control with 1% oxygen in the absence of nitrogen (●). (b) Due to practical limitations it was not possible to keep the oxygen concentration (○) constant in the batch bottles and frequent additions were required (arrows).

Nitrogenase assays

The N_2 -fixing *M. fumariolicum* cells described above were tested for nitrogenase activity. The acetylene reduction assay showed that these growing cultures indeed possessed nitrogenase activity. After 3 h of incubation with acetylene, linear ethylene production started and continued for more than 8 h at rates of $11.1 \text{ nmol h}^{-1} (\text{mg dry weight cells})^{-1}$ for the cells growing at around 1% (v/v) O_2 . A linearly growing culture (up to 2%, v/v, O_2) showed a lower nitrogenase activity of $3.7 \text{ nmol h}^{-1} (\text{mg dry weight cells})^{-1}$. Consistently, both the growth experiments and nitrogenase activity assays showed the detrimental effects of oxygen at concentration above 1% (v/v).

The non-growing control culture with argon gas, where oxygen concentration was kept constant at around 1% (v/v), also showed nitrogenase activity. After 3 h of incubation with acetylene, this culture showed linear ethylene production for more than 8 h at a rate of $5.5 \text{ nmol h}^{-1} (\text{mg dry weight cells})^{-1}$. This activity represents the induction of the nitrogenase. The 3 h incubation time required before linear ethylene production started was also observed for *Methylococcus capsulatus* Bath (Zhivotchenko *et al.*, 1995).

The differences in nitrogenase activity between the cultures grown at 1–2% (v/v) O_2 and below 1% (v/v) O_2 can be explained as a toxic effect of oxygen. Nitrogenase is known to be an oxygen-sensitive enzyme and can be irreversibly damaged by oxygen (Robson & Postgate, 1980). So it is plausible that part of the activity of this enzyme is lost during growth at 1–2% (v/v) O_2 , resulting in the linear growth observed.

Our results are in accordance with the concept that molecular oxygen is an important repressor of the transcription of *nif* genes (Rudnick *et al.*, 1997) and that the presence of N_2 is not needed for their activation.

N_2 -fixing chemostat cultures

In contrast to batch cultures, chemostat cultivation has the advantage of much better oxygen control, especially at low concentrations. A chemostat with medium containing 2% (v/v) fangaia soil extract (resulting in an initial ammonium concentration of 0.5 mM) was inoculated with *M. fumariolicum* SolV. In the first period of incubation (about 7 days), no medium was supplied to the chemostat; influent and effluent pumps were switched off. The oxygen concentration in the medium in the chemostat was controlled at 0.5% saturation. Exponential growth was observed for 2 days. The maximum growth rate (μ_{\max}) was 0.07 h^{-1} (corresponding to a doubling time of 10 h), which is identical to that of a batch culture with 5–10% (v/v) O_2 and indicates that oxygen is not limiting growth at 0.5% saturation. Growth ceased (at an OD_{600} of 0.35) when the amount of ammonium from the fangaia soil extract was depleted ($<5 \mu\text{M}$, Fig. 2a). After 1 day of adaptation the cells resumed exponential growth, but now

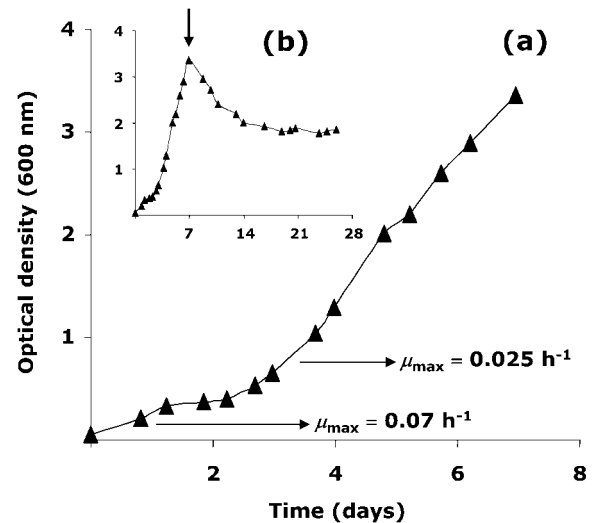


Fig. 2. (a) Growth (OD_{600}) of strain SolV in a chemostat, in nitrogen-free medium, with nitrogen gas as the only nitrogen source. After 2 days, ammonium from the fangaia soil extract had been consumed and exponential growth started. (b) On day 7 (arrow) the medium supply pump was switched on (dilution rate 0.017 h^{-1}) and the chemostat reached a steady state at day 18.

with N_2 as the only nitrogen source, at a μ_{\max} of 0.025 h^{-1} (doubling time 27 h) (Fig. 2a). The rapid onset of N_2 fixation is in contrast to the long lag phase in batch incubations, where only stationary-phase, nitrogen-depleted cells were able to start N_2 fixation. Apparently the actively growing cells in a chemostat are able to synthesize the nitrogenase protein more rapidly than the fully depleted stationary-phase cells in batch culture. After an OD_{600} of 2 was reached, exponential growth ceased and the culture showed linear growth up to OD_{600} 3.6, most likely due to CH_4 gas–liquid transfer limitations (Fig. 2a). At that time (day 7) the influent and effluent pumps of the chemostat were switched on. The chemostat was now supplied with medium, at a dilution rate of 0.017 h^{-1} (doubling time 40 h), well below the μ_{\max} observed in the batch phase. Although the soil extract added to the medium contains ammonium, the ammonium concentration in the chemostat remained below the detection limit ($<5 \mu\text{M}$). A stable steady state was reached in about 18 days (Fig. 2b). In the presence of ammonium, N_2 fixation was not expected. Cells will start to fix N_2 when the availability of nitrogen compounds becomes limiting, as was demonstrated for *Methylococcus capsulatus* Bath by Zhivotchenko *et al.* (1995). Furthermore, ammonium is an important repressor of the transcription of *nif* genes, as was observed for diazotrophic species of proteobacteria (Rudnick *et al.*, 1997).

The μ_{\max} of strain SolV under N_2 -fixing conditions was 2.8 times slower compared to growth on ammonium as nitrogen source (Pol *et al.*, 2007). This was expected, because N_2 -fixation is an endergonic process, which needs

about 16 mol ATP per N₂ molecule fixed (Dixon & Kahn, 2004). Accordingly, the growth yield of 3.5 g dry weight cells per mol CH₄ under N₂ fixing conditions was almost two times lower than the yield with ammonium as nitrogen source (6.4 g dry weight per mol CH₄; Pol *et al.*, 2007). CH₄ was converted to CO₂ according to the stoichiometry $\text{CH}_4 + 1.62 \text{O}_2 \rightarrow 0.87 \text{CO}_2 + 1.5 \text{H}_2\text{O} + 0.13 \text{CH}_2\text{O}$ (biomass). When compared to the stoichiometry of ammonium-grown cells (Pol *et al.*, 2007), a slightly higher consumption of oxygen and production of carbon dioxide was found, which coincides with the lower cell yield.

N₂ fixation by cells of strain SolV grown in chemostat cultures was confirmed by nitrogenase activity assays. The incubation with acetylene showed linear ethylene production for more than 4 h at a rate of 47.4 nmol h⁻¹ (mg dry weight cells)⁻¹. The rate is sufficient to sustain N₂ assimilation in chemostat cultures with a doubling time of 40 h. Similar nitrogenase activity values were reported for type II and type X methanotrophs (Murrell & Dalton, 1983). The activity rate of the chemostat-grown cells is four times higher than for cells growing in batch culture at <1% O₂, indicating that 1% (v/v) O₂ is still detrimental for N₂ fixation. In contrast to the inhibitory effect of ammonium on N₂ fixation in growing cultures, concentrations of ammonium up to 94 mM did not have an effect on the acetylene reduction activity of the nitrogenase. Rates were linear for more than 2 h. This was also observed for several species of proteobacteria able to fix N₂ (Rudnick *et al.*, 1997). However, Murrell & Dalton (1983) suggested that ammonium may affect the activity of nitrogenase and not the synthesis of this enzyme.

Oxygen optimum of SolV nitrogenase activity

The nitrogenase assay performed under 1% (v/v) O₂ resulted in nonlinear ethylene production, with both batch-grown cells and chemostat-grown cells. Fig. 3(a) shows ethylene production by batch-grown cells with pulse-wise addition of small amounts of oxygen. The effect of oxygen concentration on ethylene accumulation is clearly visible: e.g. addition of oxygen at day 3 slowed down production while the decreasing oxygen concentration from this point onwards resulted in an increase. This effect was explained by oxygen inhibition and was similar to the oxygen effect on growth in the N₂-fixing batch incubations described above.

To determine the optimum oxygen concentration for the SolV nitrogenase activity in whole cells, acetylene and oxygen in the headspace were set to be 2% (v/v) and 0.1–8% (v/v), respectively. The optimal oxygen concentration for SolV nitrogenase activity was found to be 0.5% (v/v) (Fig. 3b). At lower oxygen concentrations the activity decreased, possibly due to oxygen limitation for energy generation. Nitrogenase activity was still observed at oxygen concentrations up to 1% (v/v), but at oxygen concentrations above 2% (v/v) no nitrogenase activity was detected. This is in agreement with the results of batch incubations, where no growth was observed at head-space oxygen concentrations above 2% (v/v) (Fig. 1a) and favours the 0.5% O₂ saturation we used for the chemostat culture liquid medium as being optimal for growth under N₂-fixing conditions. The oxygen requirement for nitrogenase activity for other methanotrophs was 6% (v/v) for *Methylococcus capsulatus* Bath, 2% (v/v) for *Methylosinus* strain 6 and 0.5–1% (v/v) for *Methylocystis* strain T-1

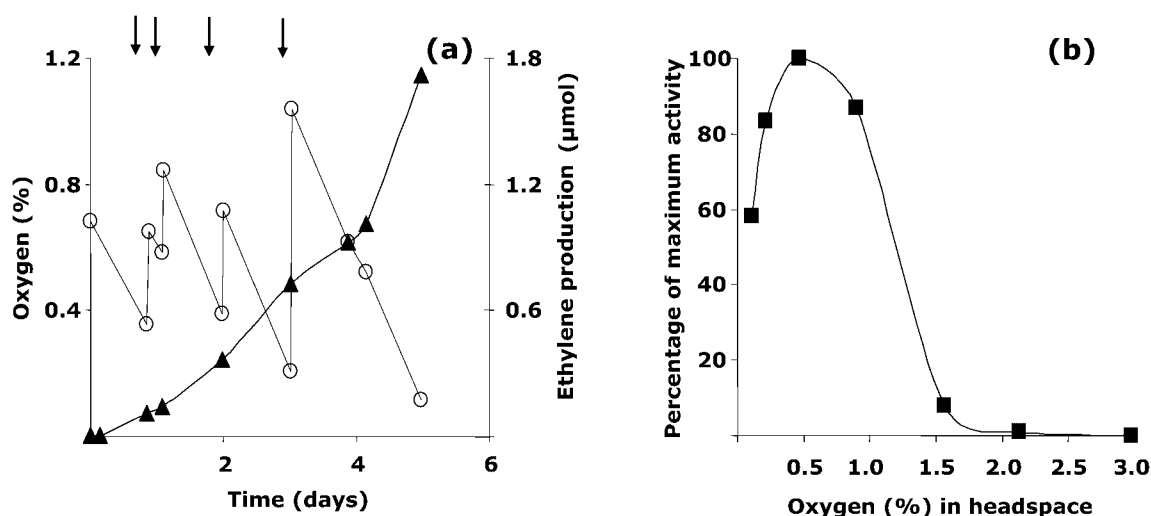


Fig. 3. (a) Effect of oxygen concentration (○) on nitrogenase activity (▲) of a N₂-fixing batch culture of strain SolV. Additions of oxygen are indicated by the arrows. (b) Cell suspensions of N₂-fixing strain SolV were incubated at different oxygen concentrations, with methanol as the oxidizable carbon substrate. Ethylene production from acetylene was measured (■); 100% activity is equal to 47.4 nmol C₂H₄ h⁻¹ (mg dry weight)⁻¹.

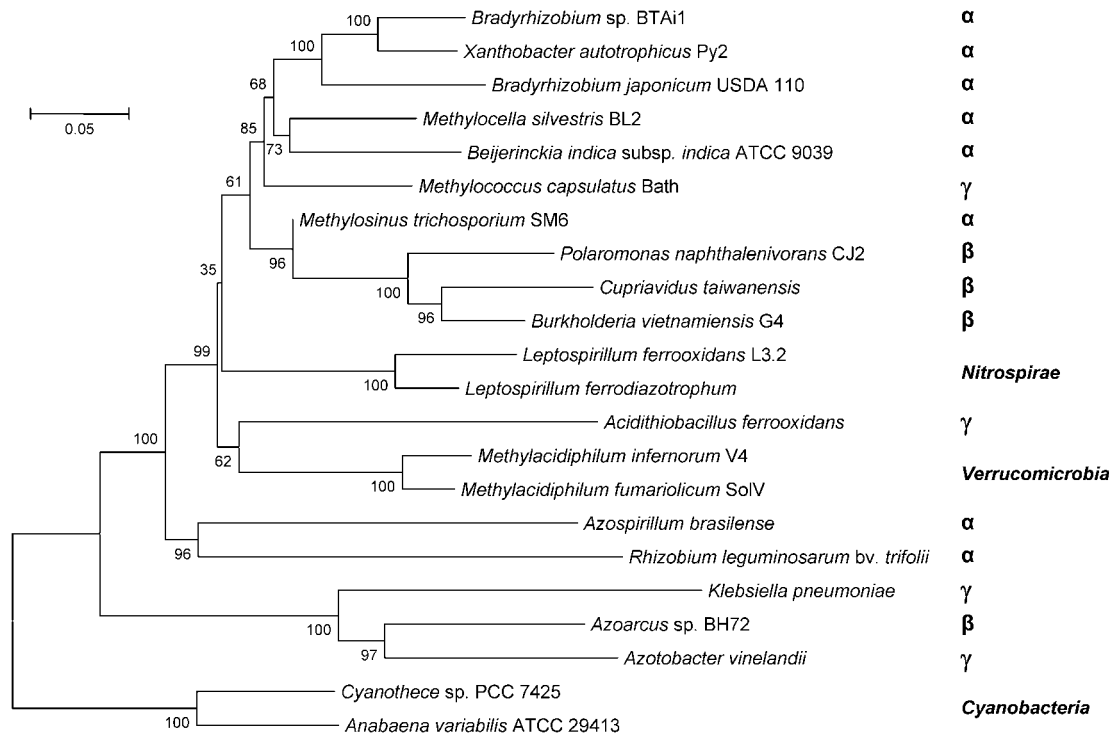


Fig. 4. Phylogenetic analysis of a concatenated set of the derived amino acid sequences of the *nifH*, *nifD* and *nifK* genes. Representative sequences were obtained from sequenced genomes (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>). Bootstrap values (500 replicates) are shown at the branches. The bar represents 5% sequence divergence. The neighbour-joining tree is drawn to scale; the evolutionary distances were computed using the Dayhoff matrix-based method and are in units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1419 positions in the final dataset.

(Zhivotchenko *et al.*, 1995; Toukdarian & Lidstrom, 1984; Takeda, 1988). If we compare the nitrogenase assay with growth experiments in nitrogen-free medium, no activity of nitrogenase was observed in assays at oxygen concentrations above 1% (v/v), while growth was observed even at 2% (v/v) O₂. This might be explained as a result of metabolic activity, which reduces the oxygen concentration in the cells.

Phylogenetic analysis of the nitrogenase

From the preliminary genome data it was clear that the important genes for N₂ fixation were present in strain SolV. The genes encoding the structural protein (*nifH*, *nifD* and *nifK*) and the genes encoding cofactor biosynthesis (*nifE*, *nifN* and *nifX*) are located in a putative operon (see Supplementary Fig. 1, available with the online version of this paper). BLASTP searches with the translated genes from the operon revealed 89 ± 6% identity with the proteins of the closely related '*Methylacidiferum infernorum*' V4. Proteins from α- and γ-proteobacterial methanotrophs showed 72 ± 10% identity. The Fe-protein from strain SolV, encoded by *nifH*, was shown to possess the cysteines involved in 4Fe–4S cluster coordination and the residues of

the two Walker motifs (Howard & Rees, 1996). The other two structural proteins, encoded by *nifD* and *nifK*, also contain the conserved residues depicted by Howard & Rees (1996).

Based upon NifH phylogeny the verrucomicrobial gene product can be grouped with Cluster I NifH proteins (Zehr *et al.*, 2003; Supplementary Fig. S2). This cluster contains the conventional Mo-containing NifH proteins. The nitrogenase is believed to be an evolutionarily conserved protein complex (Howard & Rees, 1996). The high degree of sequence similarity among nitrogenase proteins may suggest either an early origin or lateral gene transfer among prokaryotic lineages (Postgate & Eady, 1988). Although the early origin is supported by phylogenetic analysis of large sets of nitrogenase and 16S rRNA gene sequences (Postgate & Eady, 1988; Young, 1992), comparative genomics analysis supports a scenario in which nitrogenases could have been dispersed by lateral gene-transfer mechanisms. The relation of verrucomicrobial nitrogenase to that of the Archaea and Proteobacteria was investigated by analysis of a concatenated set of the translated structural genes *nifH*, *nifD* and *nifK*, taken from representative available bacterial genome sequences. Two cyanobacteria were chosen as an outgroup. The neighbour-joining phylogenetic tree (Fig. 4)

calculated from the alignment showed that the verrucomicrobial nitrogenases group with those of Proteobacteria and acidophilic *Leptospirillum* species, the latter being members of the phylum *Nitrospirae* that inhabit acid mine drainage. This finding seems to be more supportive of the lateral gene transfer scenario also in view of the grouping in the tree with a proteobacterial acidophile, *Acidithiobacillus ferrooxidans*. Based on growth experiments and the acetylene reduction assay, diazotrophy was demonstrated for *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (Mackintosh, 1978; Norris *et al.*, 1995). It is believed that the acidophilic biofilms in acid mine drainage receive limited fixed carbon and nitrogen from external sources and therefore have to fix atmospheric CO₂ and N₂ (Tyson *et al.*, 2005). For the Solfatar ecosystem less is known about the nitrogen availability. We have observed a high variety of ammonium concentrations in soil extract (see above).

Conclusion

'*Methylacidiphilum fumariolicum*' SolV was able to fix N₂ under low oxygen concentration (0.5% O₂ saturation) in chemostat cultures at a dilution rate of 0.017 h⁻¹. Based on the acetylene reduction assay and the growth experiment we conclude that the nitrogenase of strain SolV is extremely oxygen sensitive compared to most other proteobacterial methanotrophs. To our knowledge, this is the first report on the physiology of N₂ fixation within the phylum *Verrucomicrobia*.

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