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# Vanadium and molybdenum requirement for the fixation of molecular nitrogen by two *Methanosarcina* strains

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**Abstract.** Nitrogen fixation of the *Methanosarcina barkeri* strains "Fusaro" (DSM 804) and "227" (DSM 1538) was found to be dependent on the presence of vanadium or molybdenum whereby molybdenum (added as $Na_2$-molybdate) was preferred to vanadium (added as $VCl_3$). Strain "227" showed less pronounced effects on diazotrophic growth with respect to vanadium and molybdenum. Rhenium ($ReCl_3$) or tungsten ($Na_2$-tungstate) could not replace vanadium or molybdenum. The optimum concentrations were found to be 2 μM for vanadium and 5 μM for molybdenum (strain "Fusaro"). This Mo optimum of methanogenesis was 10-fold higher with $N_2$ than with $NH_4Cl$ as nitrogen source. A vanadium requirement with $NH_4Cl$ could not be detected. No interferences were observed if molybdenum and vanadium were added simultaneously under diazotrophic conditions. Growth yields were smallest for strain "227" grown diazotrophically ($Y_{CH_3OH}$ = 0.6 g dw/mol in the presence of vanadium and $Y_{CH_3OH}$ = 0.9 g dw/mol in the presence of molybdenum), obviously higher for strain "Fusaro" grown diazotrophically ($Y_{CH_3OH}$ = 1.15 g dw/mol in the presence of V and $Y_{CH_3OH}$ = 1.4 g dw/mol with Mo) and highest if *M. barkeri* was grown on $NH_4Cl$ as N-source ($Y_{CH_3OH}$ = 3.4 g dw/mol with Mo, strain "Fusaro").

**Key words:** Vanadium – Molybdenum – Methanogenesis – Nitrogen fixation – Archaebacterium

Only three naturally occurring compounds have been discovered to contain vanadium as an essential element. Originally it was assumed that a fourth vanadium containing compound occurred in tunicates, called haemovanadin (cf. Meisch and Bielig 1980), but later it was thought to be an artefact (Macara 1980). It is now believed that tunicates contain vanadium dissolved at 1 M concentration as free cation (V3 +) in green-coloured vacuoles of blood cells. A model exists to explain the huge accumulation whereas the role of vanadium in tunicates remains mysterious (Macara 1980).

The first vanadium compound isolated was the low molecular weigth substance amavadin of the mushroom *Amarita muscaria* (Bayer and Kneifel 1972). Its structure was finally confirmed by total chemical synthesis (Kneifel and Bayer 1986). Recently a bromoperoxidase was discovered in several marine brown algae and the purified protein was found to contain vanadium as an essential element (de Boer et al. 1986). Also just recently a vanadium nitrogenase could be detected and purified in special Mo nitrogenase deficient mutants of the eubacteria *Azotobacter vinelandii* and *A. chroococcum* (Bishop 1986; Hales et al. 1986; Robson et al. 1986; Eady et al. 1987). A dependence of nitrogenase on vanadium in *A. vinelandii* was proposed long ago by Bortels (1936), but this paper received little recognition.

Indication that archaebacterial methanogens could also fix molecular nitrogen was first obtained by Pine and Barker (1954) with the syntrophic culture *Methanobacterium omelianskii* and later verified by several authors with axenic cultures of 5 strains of methanogens (Murray and Zinder 1984; Belay et al. 1984; Bomar et al. 1985; König et al. 1985; Magot et al. 1986; Fardeau et al. 1987).

The following investigation was carried out to improve the knowledge of trace element requirement for *Methanosarcina* (Scherer and Sahm 1981a; Scherer et al. 1983) which is one of the ecologically most important methanogens. Another intention was to find out with *Methanosarcina* as a first example the trace elements required for diazotrophic growth in this evolutionary distinct group of archaebacteria.

## Material and methods

*Organisms. Methanosarcina barkeri* strain "Fusaro" (DSM 804) and strain "227" (DSM 1538) were obtained by Dr. H. Hippe from the "Deutsche Sammlung von Mikroorganismen" (Braunschweig, FRG).

*Culture methods.* Cultivation was performed under strictly anaerobic conditions with test tubes of 5 ml medium and 5% inoculum as described previously (Scherer et al. 1983). The growth temperature was 37° C (± 0.5° C) and the initial pH value in the imidazole buffered medium was 6.35 (± 0.03). The sole carbon source for strain "Fusaro" was 160 (± 5) mM methanol (deviations between different growth series by degassing) and a mixture of 160 (± 5) mM methanol + 50 mM sodium acetate for strain "227". $CO_2$ or a carbonate source to facilitate growth were not added. This revealed an improved reproducibility of gas production being better than 0.5% for a single growth series and a duplicate determination. The medium of strain "227" was

supplemented by additional vitamins and trace elements as listed in Scherer et al. (1983). The sulfur source sulfide was added before autoclaving as freshly prepared gaseous $H_2S$ at 1.5 mM made from $Na_2S$ and HCl. 10 mM $NH_4Cl$ or $N_2$ with an overpressure of 50 kPa was applied corresponding to a potential of approximately 145 mM available N. Vanadium was added as $VCl_3$, molybdenum as $Na_2MoO_4 \times 2\ H_2O$ and tungsten as $Na_2WO_4 \times 2\ H_2O$. Molybdate and tungstate represent stable anions whereas the vanadyl cation V 3+ and V 4+ is only stable at pH below 2 and under anaerobic conditions (Macara 1980). Rhenium was dissolved as elementary powder in $H_2O_2$ and subsequently reduced by $TiCl_3$ with resazurin as indicator for oxygen. Iron was added as 50 µM ferrous ammonium sulfate (replaced by ferrous citrate if $N_2$ was the nitrogen source) together with 50 µM ferrous citrate and 0.85 mM L-cysteine (Scherer and Sahm 1981b). An accurately degassed and separately heated iron-cysteine stock solution was a prerequisite to diminish precipitations in the culture medium after its autoclaving. It was carefully autoclaved for only 30 min (overall time) to diminish further precipitations. Culture tubes (157 × 12 mm inside) made from quartz glass were siliconized one time before use by a commercial silicone-isopropanol solution (Serva 35130, Heidelberg). Culture tubes stood upright without stirring during experiments. Chemicals were of "ultrapure grade" (Merck, Darmstadt) and the only used gases were argon (99.998%) and nitrogen (99.996%) without any treatment to eliminate impurities of oxygen (Linde AG, München). The water used was of "ultrapure" quality comparable to that obtained by a Millipore (Bedford, MA, USA) equipment (SG Wassertechnik, Hamburg). The produced gas ($CH_4 + CO_2$) was measured in duplicate assays as described previously (Scherer and Bochem 1983). Amounts of produced gases were not corrected by the small amounts of dissolved $CO_2$ (pK = 6.25, acidic growth conditions), of consumed $N_2$ and of losses by gas measurements (appr. 10%). For growth yields the cell protein was determined and multiplied with a factor of 2.0 to obtain the corresponding dry weight (Scherer and Sahm 1981b). For methanol determinations see also Scherer and Sahm (1981b).

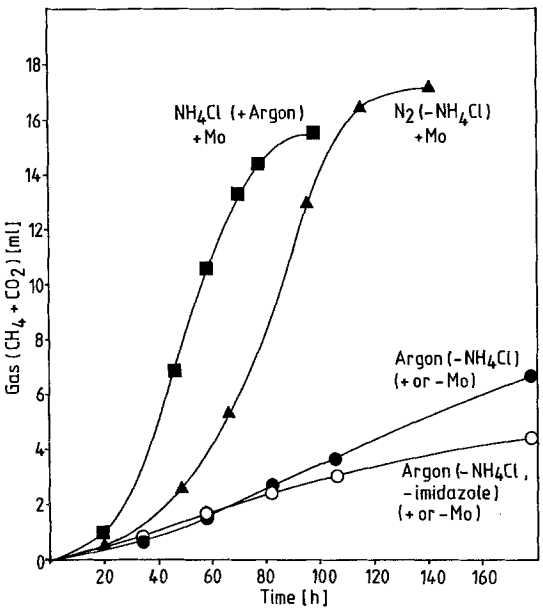


**Fig. 1.** Representative growth curves of *Methanosarcina barkeri* "Fusaro" grown on methanol in a defined medium (5.25 ml) showing the general differences between growth on $NH_4Cl$ and diazotrophic growth on $N_2$. In earlier experiments it was found that gas production correlates with growth. ■——■ Standard medium with 10 mM $NH_4Cl$ and 0.5 µM Mo (−V). Gas phase was argon (without $CO_2$). ▲——▲ Standard medium with 150 kPa $N_2$ (without stirring) as gas phase and nitrogen source and 5.0 µM molybdenum (−V; optimum concentration). Precultures on $NH_4Cl$. ●——● Control experiment without $NH_4Cl$ and $N_2$ as nitrogen source. The gas phase was argon (without $CO_2$). No significant difference could be seen between − or + 0.5 µM Mo in the standard medium. Preculture on 10 mM $NH_4Cl$. ○——○ Control experiment as above with argon as gas phase (−$NH_4Cl$, −$N_2$), but without the 40 mM imidazole buffer of the standard medium. No significant difference could be seen between − or + 0.5 µM Mo in the medium. The same preculture as above was used

## Results

In Fig. 1 complete growth curves of strain "Fusaro" are pictured demonstrating that this strain could use molecular dinitrogen as nitrogen source but preferring ammonium chloride. The found optimum concentration for molybdenum (see Fig. 3A) was applied. No lag phases could be detected although strain "Fusaro" has been cultivated continuously for many years with $NH_4Cl$ under a pure argon phase. With molecular dinitrogen as nitrogen source the length of growth periods was elongated from about 100 h to 140 h.

In Fig. 1 two control experiments are also shown demonstrating that in the special imidazole buffered medium under an argon atmosphere the 40 mM imidazole present cannot act as nitrogen source to sustain normal growth, but the imidazole was apparently responsible for a slight increase of methanogenesis. The creeping increase of methane production continued until 450 h whereas without imidazole in the culture medium methanogenesis stagnated at 250 h. The protein content after 400 h was 16 mg/l with and 12 mg/l without imidazole. Presence or absence of molybdenum had no influence. This indicated a slight availability of imidazole as nitrogen source not seen if $NH_4Cl$ was used at the standard concentration of 10 mM (data not shown). However, the zero values of growth and methanogenesis without $NH_4Cl$ or $N_2$ as nitrogen sources were presumably due to the inoculum procedure used. As cells contain approximately 13% N related to protein (Scherer et al. 1983) and the observed protein content with 10 mM $NH_4Cl$ was 270 mg/l, only 2.5 mM $NH_4Cl$ was consumed during one growth period. That means that with 5% inoculum 0.375 mM $NH_4Cl$ could be introduced yielding under optimal conditions a maximum of 40 mg/l protein.

Figure 2 shows growth curves of *Methanosarcina barkeri* strain "Fusaro" under diazotrophic conditions demonstrating that molybdenum was superior to vanadium to accomplish nitrogen fixation. The control growth curve without molybdenum or vanadium was comparable to that in the presence of argon being supplemented with or without molybdenum as presented in Fig. 1. As outlined above growth and methanogenesis in the absence of Mo and V and without addition of $NH_4Cl$ was mainly caused by $NH_4Cl$ of the inoculum. On the other hand if $NH_4Cl$ was twice omitted in subsequent transfers the resulting inoculum

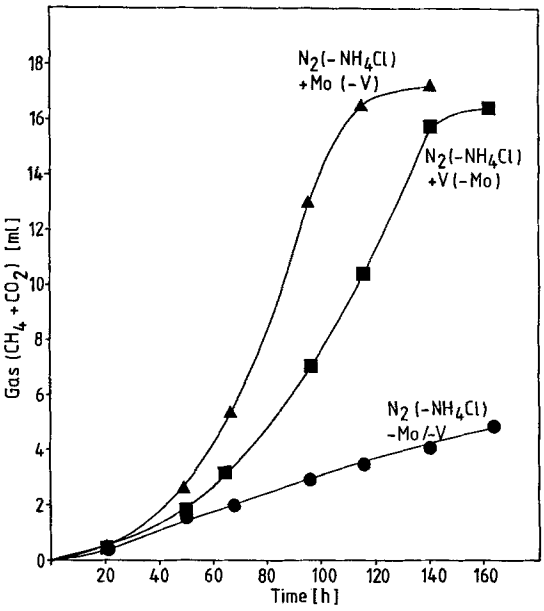


**Fig. 2.** Representative growth curves of *M. barkeri* "Fusaro" grown on methanol in a defined standard medium (5.25 ml) showing the general influences of molybdenum or vanadium on diazotrophic growth. ▲——▲ Diazotrophic growth with 150 kPa $N_2$ as gas phase (without stirring) in the presence of 0.5 μM molybdenum (−V). Standard medium as in Fig. 1A. Precultures on $NH_4Cl$. ■——■ Diazotrophic growth as above with 1.0 μM vanadium (−Mo). ●——● Diazotrophic growth as above, but in the absence of molybdenum or vanadium

culture was too weakened to obtain reproducible results. The length of growth periods reached from 190−300 h (Mo dependence) and from 230−300 h (V dependence). Similar results were obtained for *M. barkeri* "227" leading to growth periods of 140−260 h (Mo dependence) or 160−280 h (V dependence).

Figure 3A presents the optimum concentration of molybdenum for nitrogen fixation respectively methanogenesis and growth (protein increase) under diazotrophic conditions of *M. barkeri* "Fusaro". Somewhat weaker effects were obtained with strain "227" (data not shown). The optimum concentration of molybdenum was 5 μM for growth and methanogenesis of strain "Fusaro" and above 10 μM for strain "227".

The amounts of gas ($CH_4$ + $CO_2$) produced during consumption of methanol parallelled in general with the height of cell protein. However, if the Mo concentrations were altered the total produced gas at the end of growth was highest at very low levels of Mo, whereas the maximum value of cell protein was at significantly higher concentrations of Mo (Fig. 3A).

Figure 3B demonstrates again diazotrophic growth of *M. barkeri* strain "Fusaro", this time illustrating the dependence of nitrogen fixation on vanadium. To reveal this requirement molybdenum had to be absent. The optimum concentration of vanadium was approximately 2 μM for growth and methanogenesis of strain "Fusaro" and also of strain "227" (data not shown).

Corresponding experiments with rhenium and tungsten in the range of 0−5 μM showed that these elements could not replace vanadium or molybdenum.

The protein contents of centrifuged *M. barkeri* cells after growth (mg/l culture liquor) were smallest for strain "227" grown diazotrophically (maximum was nearly 50 mg/l = $Y_{CH_3OH}$ 0.6 g dw/mol in the presence of vanadium and 70 mg/l = $Y_{CH_3OH}$ 0.9 g dw/mol in the presence of molybdenum), clearly higher for strain "Fusaro" grown diazotrophically (appr. 90 mg/l = $Y_{CH_3OH}$ 1.15 g dw/mol in the presence of V and appr. 110 mg/l = $Y_{CH_3OH}$ 1.4 g dw/mol with Mo) and highest if *M. barkeri* was grown on $NH_4Cl$ as N-source (appr. 270 mg/l = $Y_{CH_3OH}$ 3.4 g dw/mol, strain "Fusaro"). With $NH_4Cl$ as nitrogen source the differences of protein contents respectively growth yields were not very pronounced at different molybdenum concentrations. Under these conditions the optimum of Mo was determined to be 0.5 μM. At higher Mo concentrations a slight inhibition of growth could be observed (range 5−15% at 1−5 μM, data not shown).

The final pH values of the cultures ranged between 4.5−4.6 for strain "Fusaro" grown on methanol with $NH_4Cl$ and between 5.8−5.9 grown diazotrophically (with V or with Mo) as no HCl could be released any longer by consumption of $NH_4Cl$ as nitrogen source. Strain "227" was grown mixotrophically (Scherer and Sahm 1981b) and the acetate consumption compensated for the pH decrease. The final pH values of cultures of strain "227" with $N_2$ as N-source ranged between 6.4−6.4 if vanadium was present and between 6.3−6.4 in the presence of molybdenum.

Further experiments with suboptimal concentrations of vanadium added simultaneously with molybdenum are shown in Fig. 4. Methanogenesis of strain "Fusaro" was not significantly improved when supplemented with 0.2 μM V plus 0.2 μM Mo instead of 0.2 μM Mo given alone (Fig. 4). The same was found for protein contents (data not shown). Therefore, cometabolism of both elements was not evident and no interferring effects were exhibited. However, it must be mentioned that suboptimal concentrations had to be used to find out any influence of V in the presence of Mo. Therefore, the possible magnitude of a response was reduced.

## Discussion

Figures 2, 3B, and 4 demonstrate for the first time that *Methanosarcina barkeri* strain "Fusaro" can fix molecular nitrogen in the presence of vanadium instead of molybdenum. The same was found for strain "227". This and the fact that the requirement for vanadium was not detectable during growth with ammonium chloride as N-source support the assumption that *M. barkeri* is able to build a vanadium nitrogenase as found for special Mo-nitrogenase negative mutants of the eubacterium *Azotobacter* (Bishop 1986; Hales et al. 1986; Eady et al. 1987). Furthermore, Figs. 1, 3A, and 4 prove for the first time that molybdenum can accomplish nitrogen fixation of archaebacterial methanogens as was documented for eubacteria over 50 years ago (Bortels 1936).

Some indications for Mo requirement under diazotrophic conditions of *Methanococcus thermolithotrophicus* were mentioned without details recently by Magot et al. (1986). Interestingly these authors detected homologies of the *nifH* gene (nitrogenase Fe protein) between *Klebsiella* and *Anabaena*, and 14 methanogenic strains by Southern hybridization techniques. A weaker homology to *nifD* and *nifK* (nitrogenase MoFe protein) was found only in 5 strains (*M. barkeri* "227" without *nifD*), but no homologous

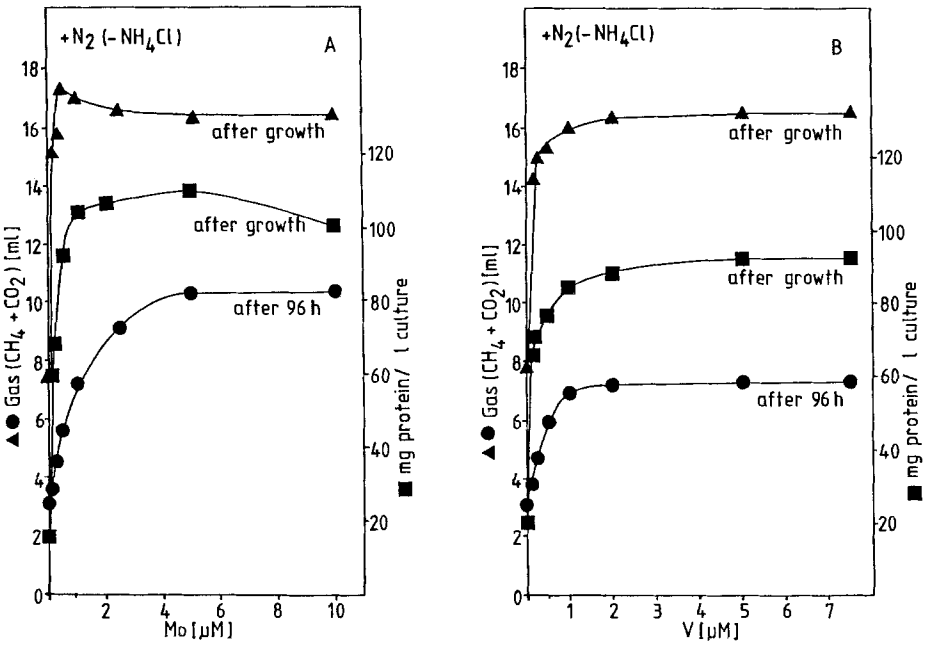


**Fig. 3A, B**
Diazotrophic growth of *M. barkeri* "Fusaro" and its dependence on different concentrations of molybdenum (**A**) or vanadium (**B**) in the defined standard medium. The following additions were applied: 0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 µM Mo as well as 0, 0.1, 0.5, 1.0, 2.0, 5.0 and 7.5 µM V. ●——● Gas production ($CH_4 + CO_2$) of 5.25 ml medium after 96 h. The time 96 h (cf. Fig. 1) was chosen to exhibit more dynamics than after growth. ▲——▲ Gas production after growth if the stationary phase was reached. ■——■ Protein yields per l culture medium. Protein yields were measured after growth had ceased

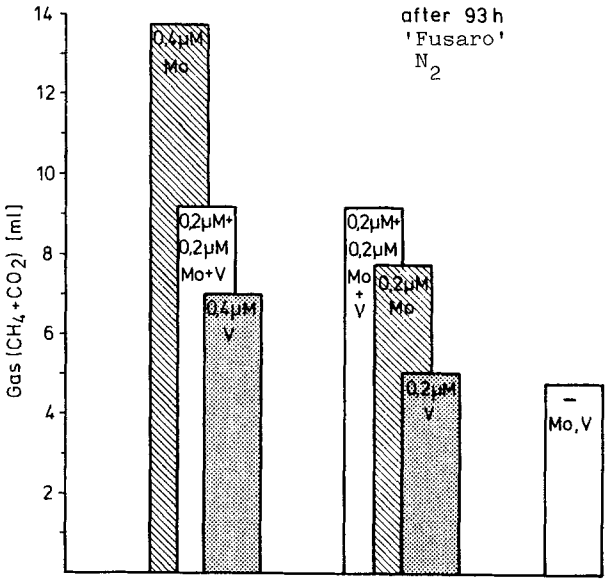


**Fig. 4.** Diazotrophic growth conditions of *M. barkeri* "Fusaro" and the dependence of gas production ($CH_4 + CO_2$, after 93 h) on the simultaneous presence of vanadium and molybdenum. Conditions as in Fig. 1

polypeptides could be discovered by anti-MoFe protein antibodies.

The ability of *Methanosarcinae* to fix nitrogen has already been reported (Murray and Zinder 1984; Bomar et al. 1985), but diazotrophic growth failed with *Methanococcus voltae* (Magot et al. 1986) and presumably with *Methanosarcina* strain "TM1" (Murray and Zinder 1984). The experiments further show that under the above defined growth conditions strain "Fusaro" was a better candidate for nitrogen fixation than strain "227". This could be explained by the observation that growth of strain "227" with $NH_4Cl$ was also slower, although growth was supported by addition of acetate and additional vitamins and trace elements. The published optimum of 0.5 µM Mo for growth on $NH_4Cl$ of strain "Fusaro" could be confirmed exactly (Scherer and Sahm 1981a).

In contrast to *M. thermolithotrophicus* which showed lag phases of 2–3 weeks (Belay et al. 1984) no lag phases could be recognized if growth was shifted from $NH_4Cl$ to $N_2$ as N-source. Such lag phases were not confirmed by Magot et al. (1986) either who worked with *M. thermolithorophicus* and *Methanobacterium ivanovi.*

As was recently published for the *M. barkeri* strains "227" (Murray and Zinder 1984) and "Fusaro" (Bomar et al. 1985), for *M. thermolithotrophicus* (Magot et al. 1986) and in continuous culture experiments for *M. thermolithotrophicus* and *M. thermoautotrophicum* strain "Marburg", the growth yields with $NH_4Cl$ were obviously higher than with $N_2$ as N-source (Fardeau et al. 1987). These findings could be validated with the present study. Moreover it was found that for diazotrophic growth of the two *M. barkeri* strains growth yields in the presence of molybdenum were substantially higher than in the presence of vanadium: $Y_{CH_3OH}$ = 1.15 (strain "227") and 1.4 g dw/mol (strain "Fusaro") with Mo and $Y_{CH_3OH}$ = 0.6 (strain "227") and 0.9 g dw/mol (strain "Fusaro") with V in the medium. The same tendency with similar percentages could be observed if the vanadium nitrogenase of *A. vinelandii* was compared with the molybdenum enzyme in terms of $N_2$ reduction. The vanadium containing nitrogenase (component 1) showed only about 63% activity of the corresponding molybdenum containing enzyme (Hales et al. 1986). A more unfavourable relation to nitrogen fixation was found for the vanadium containing nitrogenase of *A. chroococcum* (Robson et al. 1986; Eady et al. 1987).

The data of the present study reveal that at "zero"-concentrations of V and Mo remarkable gas production and cell growth occur under diazotrophic conditions. It has been already pointed out that this phenomenon could be explained by $NH_4Cl$ contaminations of the inoculum. Just recently a third type of nitrogenase was reported, containing iron only, with no molybdenum or vanadium (Chisnell et al. 1988). It cannot be excluded that such a nitrogenase is also

active in methanogens, although its activity in *A. vinelandii* was only a few per cent of the MoFe enzyme.

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