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Starvation Alters the Apparent Half-Saturation Constant for Methane in the Type II Methanotroph *Methylocystis* Strain LR1

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When cells of a type II methanotrophic bacterium (*Methylocystis* strain LR1) were starved of methane, both the $K_{m(app)}$ and the $V_{max(app)}$ for methane decreased. The specific affinity (a^o_s) remained nearly constant. Therefore, the decreased $K_{m(app)}$ in starved cells was probably not an adjustment to better utilize low-methane concentrations.

Microbial oxidation of atmospheric methane (CH_4) takes place in most aerobic upland soils (5, 10). Because these soils exhibit a lower half-saturation constant [$K_{m(app)}$] for CH_4 than do pure cultures of methanotrophic bacteria, it has been postulated that the active bacteria are unknown species. These have been popularly dubbed "high-affinity" methane oxidizers (5, 10). Recently, a novel group of *pmoA*-like sequences was detected in several soils which oxidize atmospheric CH_4 (11, 12), and incubation of soils under $^{14}CH_4$ resulted in the labeling of signature phospholipid fatty acids which differed from those of known type II methanotrophs (18). It is therefore likely that as-yet-uncultured species are involved in atmospheric CH_4 uptake. However, it remains unknown whether atmospheric CH_4 oxidation is limited to particular species and whether these possess a specialized high-affinity CH_4 oxidation enzyme.

We previously demonstrated that high-affinity CH_4 oxidation is probably not limited to uncultured methanotrophic groups. We enriched a methane-oxidizing bacterium (strain LR1) from an organic soil and identified it based on 16S rDNA, *pmoA*, and *mxaF* gene sequences as a type II methanotrophic species of the *Methylosinus*/*Methylocystis* cluster (8). Mixed cultures containing strain LR1, when grown under <275 ppm volume CH_4, had a low $K_{m(app)}$ for CH_4 (56 to 188 nM) similar to the value measured in soil. This increased to >1 μM when cells were grown under >1% CH_4. In the present study, we investigated the kinetics of the isolated bacterium (culture is available upon request). Instead of the time-consuming process of growing the organism under low CH_4 mixing ratios, we tested the effect of starving cells of CH_4.

Kinetics of strain LR1. Culture was grown in liquid nitrate-mineral salts (NMS) medium (8) under 10% CH_4. Purity was controlled microscopically and by plating onto NMS agar, R2A agar, 10% strength Nutrient Agar, and 10% strength AC Broth (Difco). After 1 to 2 months, the culture was diluted to about 10^9 cells ml^{-1} with 0.5 mM phosphate buffer (pH 6.0), and 7.5-ml amounts were added to 13-ml serum vials. The vials were capped with sterile butyl rubber stoppers and incubated with gentle shaking (6 rpm) at 25°C without added CH_4. After 1 to 2 weeks, some vials were injected with CH_4 to a final mixing ratio of 1% and incubated for a further 24 h ("unstarved"). Others remained without CH_4 ("starved"). Cell counts were made using a Neubauer chamber and showed that no population growth occurred during the 24-h incubation with 1% CH_4 (data not shown).

For determination of kinetic properties, CH_4 was injected into these vials to final mixing ratios ranging from 5 to 1,500 ppm volume. The unstarved vials still contained >0.5% CH_4 after 24 h and were first flushed well with air. Vials were shaken at 280 rpm. Starting 5 min after CH_4 addition and at 30-min intervals thereafter, CH_4 was measured by gas chromatography-flame ionization detection (8). Methane oxidation rates and kinetic parameters were estimated as previously described (8).

Unstarved cells had both a higher $K_{m(app)}$ and a higher $V_{max(app)}$ for CH_4 than did starved cells (Table 1). Addition of KCl may also have decreased the $K_{m(app)}$. The increase of both $K_{m(app)}$ and $V_{max(app)}$ in unstarved culture was about 1 order of magnitude, and the specific affinity (a^o_s) ($V_{max(app)}/K_{m(app)}$) therefore remained nearly constant. The specific affinity is the initial slope of the hyperbolic curve, or the pseudo first-order rate constant, and directly indicates how rapidly the culture metabolized limiting substrate (4). At low CH_4 concentrations, the rate of CH_4 uptake was therefore similar in starved and unstarved culture.

These results extend our previous observations on strain LR1 by demonstrating that (i) the $K_{m(app)}$ can vary in pure culture, (ii) starvation of CH_4 decreases the $K_{m(app)}$, (iii) the specific affinity (a^o_s) changes little with $K_{m(app)}$, and (iv) $K_{m(app)}$ varies in rapid (<2 h) tests without added chloramphenicol (previous tests were run over several days and chloramphenicol was necessary to prevent enzyme production). As previously discussed (8), the variable $K_{m(app)}$ could have resulted because type II methanotrophs possesses different forms of methane monooxygenase (MMO): a particulate (pMMO) and a soluble (sMMO) form. Multiple catalytic forms of pMMO also exist, depending on Cu availability (15, 17). However, while the $K_{m(app)}$ values of these MMOs are different (10, 15, 21), all measured values in pure culture are above 0.8 μM. The values measured in strain LR1 are as low as 56 to 336 nM (present results and reference 8).

Multiple enzymes may be responsible for the variable $K_{m(app)}$ in LR1, but because the a^o_s per cell remained constant, it cannot be concluded that a high-affinity enzyme was induced to better utilize limiting CH_4. This possibility is consistent with the data only if reactivation of inactive cells and expression of a lower-affinity enzyme compensated for each other in the unstarved culture and caused the a^o_s to remain constant. However, if this were so, a biphasic kinetic curve should have been

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TABLE 1. Kinetic coefficients of starved and unstarved cultures of *Methylocystis* strain LR1 in several trials[a]

Trial	Measurement time (h)	$K_{m(app)}$ (nM CH_4)	$V_{max(app)}$ (10^{-9} nmol cell^{-1} h^{-1})	a^o_s (10^{-9} ml cell^{-1} h^{-1})	
				$V_{max(app)}/K_{m(app)}$	Regression
Trial 1					
Cells starved 12 days	1	298	2.61	8.75	4.65
Starved + KCl[a]	1	138	1.22	8.84	5.27
Unstarved	1	3,170	18.7	5.90	4.33
Trial 2					
Cells starved 34 days	2	281	0.935	3.32	2.09
Starved + KCl[b]	2	226	0.380	1.68	0.95
Unstarved	1.5	12,600	27.8	2.21	2.12
Trial 3					
Cells starved 10 days	2	336	2.97	8.88	4.73
Fresh[c]	2	2,190	13.27	6.08	4.39
Trial 4					
Cells starved 19 days	2	329	0.779	2.36	1.39
Unstarved	1.5	3,400	21.7	6.38	4.81

[a] Each calculation was based on data from 12 to 54 individual vials. Michaelis-Menten kinetic coefficients are shown, as well as the specific affinity (a^o_s) either calculated ($V_{max(app)}/K_{m(app)}$) or estimated by linear regression of the CH_4 oxidation rate versus the dissolved CH_4 concentration for all data points less than $K_{m(app)}$.
[b] KCl was added to a final concentration of 250 mM immediately before test.
[c] Cells were not preshaken and unstarved, but were taken directly from growing stock culture containing >1% CH_4.

evident, and this was never observed. A better explanation for our results is that measured $K_{m(app)}$ values do not always represent true, constant enzyme properties. Because of diffusion limitation in experimental systems, many reported $K_{m(app)}$ values are gross overestimates (14). In order to control for this in our experiments, the CH_4 oxidation rate constants in the linear portion of the hyperbolic curve were also often measured with culture diluted 50%. These rate constants were close to 50% of the rate constants in undiluted culture (in six cases, rate constants were 42, 54, 61, 73, 74, and 81%), indicating that there was only a slight limitation in CH_4 movement from the gas phase to the liquid phase. We also used the minimum incubation times which yielded reproducible data (<2 h) to estimate CH_4 oxidation rates. To illustrate the effect of longer incubation times, we measured an initial 0 to 2 h rate at five CH_4 concentrations in a 10-day-starved culture, and then after 4 h (during which the CH_4 declined <40%) reinjected enough CH_4 to bring each vial back to its initial CH_4 concentration and measured a 4 to 6 h rate (Fig. 1). The CH_4 oxidation rate increased with time, most strikingly at the higher CH_4 concentrations. This trend leads to an overestimation of the $K_{m(app)}$ with increasing incubation times.

Although we minimized these methodological problems, there are further ways in which an apparent K_m in a complex system can vary. The $K_{m(app)}$ of MMO for CH_4 is affected by the CH_4 association and dissociation constants, the rate of CH_4 diffusion across the cell envelope, and the concentrations of cosubstrates (O_2 and reductant). One explanation for the lower $K_{m(app)}$ in starved cells is low reductant availability. The mechanism of pMMO is not well elucidated, but sMMO follows a catalytic sequence in which O_2, CH_4, and NADH sequentially bind (9, 20). If in starved cells CH_4 binds normally but the overall catalytic cycle is slowed by NADH limitation, the rate-limiting step may cease to be the association and dissociation of CH_4 to the enzyme (i.e., the affinity constant) and instead become the reaction rate (i.e., the kinetic constant). In such a case, both the $V_{max(app)}$ and the $K_{m(app)}$ for CH_4 decrease (19). To illustrate this, the following reaction diagram has been simplified to consider only CH_4 as a reactant. The binding of O_2 and NADH and the formation of methanol are considered by the rate constant k_p:

$$\mathrm{MMO_{ox}} + \mathrm{CH_4} \underset{k_{(-s)}}{\overset{k_s}{\rightleftharpoons}} \mathrm{MMO\text{-}CH_4} \underset{k_{(-p)}}{\overset{k_p}{\rightleftharpoons}} \mathrm{MMO_{ox}} + \mathrm{CH_3OH}$$

Here, $K_{m(app)} = [k_{(-s)} + k_p]/[k_s + k_{(-p)}]$. Limitation of reductant will decrease the reaction rate constant k_p and in turn decrease $K_{m(app)}$ (i.e., cause a higher apparent affinity). The above model is a very simple explanation of the observed variability. The truth may of course be more complex.

Bender and Conrad (2) observed that incubation of various soils under 20% CH_4 increased methanotrophic $K_{m(app)}$ and $V_{max(app)}$ values by 1 to 3 orders of magnitude, but increased methanotrophic cell counts only 3- to 10-fold. When specific affinities were calculated (Table 2), two of these soils (a

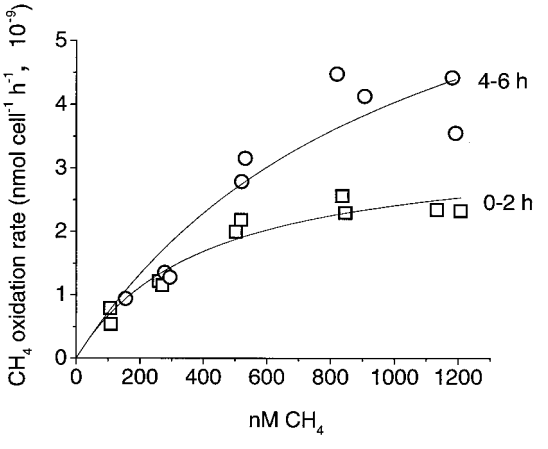

FIG. 1. CH_4 oxidation rates at five CH_4 concentrations in a 10-day-starved culture of *Methylocystis* strain LR1, measured 0 to 2 h (□) and 4 to 6 h (○) after adding CH_4.

TABLE 2. Michaelis-Menten apparent half-saturation constants for CH_4 oxidation in three soils, and calculated specific affinities from data given in reference 2[a]

Sample	$K_{m(app)}$ (nM CH_4)	a^o_s (10^{-9} ml cell^{-1} h^{-1})
Cultivated cambisol		
Fresh	50.6	0.0039
+20% CH_4	91	0.017
	1,740	0.016
Meadow cambisol		
Fresh	49.9	0.050
+20% CH_4	12.6	0.051
	4,560	0.027
Forest luvisol		
Fresh	29.7	0.505
+20% CH_4	470	0.136
	27,900	0.025

[a] Data are given for fresh soil and soils preincubated 3 weeks under 20% CH_4. In preincubated soils, a dual kinetic was evident, with both a high-affinity (second row for each soil) and a low-affinity (third row for each soil) activity.

meadow cambisol and a cultivated cambisol) had a similar pattern as LR1—that a^o_s varied little despite large changes (30- to 100-fold) of $K_{m(app)}$. A third soil (forest luvisol) had a much lower a^o_s after enrichment than before, suggesting that a different population had become active in CH_4-enriched soil. Comparisons must be cautiously made, but these data suggest that the pattern noted in LR1 is applicable to some, but not all, soils.

It is clear from this and other work (3, 8) that the $K_{m(app)}$ for CH_4 changes with culture conditions. Nevertheless, our lowest measured $K_{m(app)}$ is still higher than the lowest measured values in soil of about 10 nM (8), so we hesitate to conclude that no true high-affinity MMO exists. Calculations based on maintenance energy requirements suggest that methanotrophic bacteria cannot survive on atmospheric CH_4 without a more efficient CH_4-oxidizing system (6). However, soil methanotrophs may not consume only atmospheric methane but also alternate substrates, such as methanol (3, 13), or CH_4 produced in anaerobic soil microsites (1, 7, 16, 22). Our present results show that the observed high-affinity activity in soil cannot in itself be taken as proof that the responsible bacteria are novel oligotrophic species with a specialized form of MMO.

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