Phosphate Inhibits Acetotrophic Methanogenesis on Rice Roots

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The contribution of acetate- and \( \text{H}_2/\text{CO}_2 \)-dependent methanogenesis to total \( \text{CH}_4 \) production was determined in excised washed rice roots by radiolabeling, methyl fluoride inhibition, and stable carbon isotope fractionation. Addition of \( \geq 20 \) mM phosphate inhibited methanogenesis, which then was exclusively from \( \text{H}_2/\text{CO}_2 \). Otherwise, acetate contributed about 50 to 60% of the total methanogenesis, demonstrating that phosphate specifically inhibited acetotrophic methanogenesis on rice roots.

The trend toward increasing \( \text{CH}_4 \) levels in our atmosphere (5, 13) and the contribution of \( \text{CH}_4 \) to global warming (17) have raised interest in the microbial processes that contribute to the global \( \text{CH}_4 \) cycle. Flooded rice fields are an important source of atmospheric \( \text{CH}_4 \), contributing about 20% of the total \( \text{CH}_4 \) budget (9, 17). Most (about 90%) of the \( \text{CH}_4 \) emitted from rice fields is ventilated through rice plants (26). Recently, it was shown that \( \text{CH}_4 \) is produced not only in anoxic rice field soil but also directly on the roots of rice plants, which are inhabited by a methanogenic flora that is distinct from that in rice field soil (14, 15, 16, 21). The relative contribution of the methanogens on rice roots versus those in the soil to the total emission of \( \text{CH}_4 \) from rice fields is unknown. However, the microbial root flora possibly contributes a significant portion, since up to 50% of the \( \text{CH}_4 \) that is emitted from rice fields originates from plant photosynthates that are excreted from rice roots (23) and about 3 to 6% of the photosynthetically fixed \( \text{CO}_2 \) is converted to \( \text{CH}_4 \) and emitted (10).

Recently, we have shown that excised washed rice roots that are incubated under anoxic conditions convert \( \text{CO}_2 \) to acetate, propionate, and \( \text{CH}_4 \) (7). The produced acetate, on the other hand, have so far only been observed in paddy soil, but not on rice roots (15, 16). \( \text{CH}_4 \) production accelerated only occasionally, especially upon prolonged incubation or during incubation of roots without buffer, and only during these rare events did acetate-dependent methanogenesis operate (21).

We therefore hypothesized that the failure to detect acetotrophic methanogenesis in rice root preparations may be due to the specific incubation conditions used. Acetotrophic methanogens are, indeed, sensitive to incubation conditions. For example, acetate-dependent methanogenesis in anoxic paddy soil is activated when the soil slurry is stirred with a magnetic bar, which destroys \textit{Methanosarcina} cells (11). Since inactivation by mechanical forces was unlikely for the root incubations, we considered the possibility that the phosphate buffer which was used as the incubation medium was inhibitory. Here, we report that this was, indeed, the case.

The growth of rice plants, the preparation of rice roots, and the incubation experiments have already been described in detail (7). Briefly, rice plants (\textit{Oryza sativa}, var. Roma, type japonica) were grown in a greenhouse. After 12 to 14 weeks, the plants were removed from the soil and the roots were washed, cut with a razor blade, and kept under \( \text{N}_2 \). Aliquots of fresh roots (5 or 10 g) were incubated at room temperature (about 25°C) in 50 ml of anoxic sterile buffer under an \( \text{N}_2 \) atmosphere using stoppered glass bottles (150-ml volume). The buffer consisted either of demineralized water plus 5 g of marble granules (0.5- to 2.0-mm size, consisting of \( \text{CaCO}_3 \); Merck, Darmstadt, Germany) or of phosphate buffer (50 mM \( \text{KH}_2\text{PO}_4 \), 17 mM \( \text{NaCl} \), 0.2 mM \( \text{MgCl}_2 \), pH 7.0). Gas samples (0.25 to 1.0 ml) and liquid samples (0.5 ml) were analyzed as previously described (7). In some experiments, \( \text{CH}_3\text{F} \) was added to the gas phase at a concentration of 1.0%. Radiotracer experiments were done as previously described (7) by adding 50 to 100 \( \mu \text{Ci} \) of \( \text{NaH}^{14}\text{CO}_3 \) or \( \text{Na-[2-14C]} \text{acetate} \). The fraction \( f \) of \( \text{CH}_4 \) that was produced by the reduction of \( H^{14}\text{CO}_3 \) was calculated from the specific radioactivities of \( ^{14}\text{CH}_4 \) (\( \delta_{\text{CH}_4} \)) and \( ^{14}\text{CO}_2 \) (\( \delta_{\text{CO}_2} \)) measured in the gas phase using the formula \( f = \delta_{\text{CH}_4}/\delta_{\text{CO}_2} \). The fraction of acetate carbon that was produced by reduction of \( \text{H}^{14}\text{CO}_3 \) and the fraction of \( \text{CH}_4 \) that was produced by cleavage of acetate were calculated analogously.

Stable-isotope analysis of \( ^{13}\text{C}/^{12}\text{C} \) in gas samples was performed using a gas chromatograph combustion-isotope ratio mass spectrometry (IRMS) system purchased from Finnigan (Thermoquest, Bremen, Germany). The operation principle was described previously (4, 30). The isotopes were detected in a Finnigan MAT delta plus IRMS apparatus. The \( \text{CH}_4 \) and \( \text{CO}_2 \) in the gas samples (10 to 400 ml) were separated in a Hewlett-Packard 6890 gas chromatograph operating with a Poraplot Q column (27.5-m length, 0.32-mm inside diameter, 10-µm film thickness; Chrompack, Frankfurt, Germany) at 25°C and He (99.996% purity, 2.6 ml min \(^{-1} \)) as the carrier gas. The separated gases were then converted to \( \text{CO}_2 \) in a Finnigan Standard GC Combustion Interface III and transferred into the IRMS apparatus. The working standard was \( \text{CO}_2 \) gas (99.998% purity; Messer-Griesheim, Düsseldorf, Germany) calibrated against Pee Dee Belemmite carbonate. The isotopic ratios were expressed as follows in the delta notation: \( \delta ^{13}\text{C} = 10^3 (R_{\text{sam}}/R_{\text{std}} - 1) \) with \( R = ^{13}\text{C}/^{12}\text{C} \) of the sample (\( \text{sam} \)) and the standard (\( \text{std} \), respectively. The precision of repeated analysis was \( \pm 0.2\% \) when 1.3 nmol of \( \text{CH}_4 \) was injected. The fractionation factor \( \alpha \) for \( \text{CH}_4 \) formation from \( \text{CO}_2 \) was obtained from the formula \( \alpha = (\delta ^{13}\text{CO}_2 + 10^3)/(\delta ^{13}\text{CH}_4 + 10^3) \) (35).

Both phosphate and marble granules buffered the root incubations sufficiently well to keep the pH within a physiological pH range of 6.6 to 7.1. When phosphate was added to marble-
buffered root incubations, CH₄ production was inhibited at phosphate concentrations of ≥20 mM (Fig. 1). Addition of only 10 mM phosphate, on the other hand, slightly enhanced the production of CH₄.

The roots that were incubated in 50 mM phosphate buffer in the presence of NaH¹⁴CO₃ produced ¹⁴CH₄ for about 10 days. The final CH₄ partial pressure was about 50 to 150 Pa. Methyl fluoride has been shown to preferentially inhibit acetotrophic methanogenesis (8, 14, 18). However, addition of methyl fluoride inhibited neither the production of CH₄ nor the production of ¹⁴CH₄ from NaH¹⁴CO₃, indicating that all of the CH₄ produced originated from CO₂ reduction. CO₂ was not limiting, as it increased to partial pressures of ≥2 kPa within 50 h. These observations corroborated results of earlier experiments which were also conducted with 50 mM phosphate buffer (7, 21).

By contrast, root incubations buffered with marble granules produced much more CH₄ (>5,000 Pa) during 10 days with gradually increasing rates. CO₂ was produced to partial pressures (>2 kPa within 50 h) similar to those found in the phosphate-buffered incubations. Addition of methyl fluoride partially inhibited the production of total CH₄ by about 50%, indicating that acetotrophic methanogenesis contributed substantially to CH₄ production. Production of ¹⁴CH₄ from NaH¹⁴CO₃ was also enhanced in marble-buffered incubations compared to phosphate-buffered incubations (Fig. 2A). The percentage of CH₄ produced from CO₂, which was calculated from the specific radioactivities of ¹⁴CH₄ and NaH¹⁴CO₃, was much lower in the incubations with marble granules (63% ± 9%; mean ± standard deviation [SD]; n = 12) than in those with phosphate (102% ± 8%).

Acetate was produced simultaneously and accumulated to maximum concentrations of 10 to 13 mM both with marble granules and with phosphate. Labeling experiments with NaH¹⁴CO₃ showed that CO₂ was converted to acetate (Fig. 2A). As in earlier experiments (7), a relatively large percentage of the acetate carbon originated from CO₂, both in the marble-buffered (46% ± 19%) and in the phosphate-buffered (57% ± 15%) incubations. However, the radioactive acetate was subsequently consumed in the marble-buffered incubations, presumably by conversion to CH₄, whereas it stayed constant in the phosphate-buffered incubations (Fig. 2A). Total acetate also decreased from about 10 to 4 mM until the end of incubation.

Phosphate-buffered root incubations did not convert [2-¹⁴C]acetate (Fig. 2B), as found earlier (21). Marble-buffered root incubations, on the other hand, converted [2-¹⁴C]acetate to ¹⁴CH₄ plus ¹⁴CO₂ (Fig. 2B). ¹⁴CO₂ was produced right in the beginning of the incubation but soon reached a constant level equivalent to about 8 to 10% of the initially added [2-¹⁴C]acetate. We assume that acetate was oxidized by iron-reducing bacteria during this phase, since the roots were partially encrusted with ferric iron plaques, which may have served as an oxidant. Conversion of [2-¹⁴C]acetate to ¹⁴CH₄, on the other hand, gradually increased with time, as CH₄ production did. The percentage of CH₄ produced from acetate, which was calculated from the specific radioactivities of ¹⁴CH₄ and [2-¹⁴C]acetate, was 58% ± 9%.

The increased contribution of acetate to total CH₄ production in the absence of phosphate also affected the stable iso-

**FIG. 1.** Effect of phosphate addition on CH₄ production by excised washed rice roots (10-g fresh weight) incubated in 50 ml of water with 5 g of marble granules (control). Different concentrations of phosphate were adjusted by addition of phosphate buffer. Each point is the mean ± SD of triplicate determinations.

**FIG. 2.** Production of ¹⁴CH₄ and [¹⁴C]acetate from NaH¹⁴CO₃ (A) and of ¹⁴CH₄ and ¹⁴CO₂ from [2-¹⁴C]acetate (B) by excised washed rice roots (10-g fresh weight) incubated in either 50 ml of phosphate buffer, pH 7, or 50 ml of water with 5 g of marble granules. Experiments A and B were done with different root preparations. Each point is the mean ± SD of triplicate determinations.
acetotrophic methanogenesis to total CH₄ production (Fig. 3). The stable isotope signatures of CH₄ and CO₂ were measured in both marble- and phosphate-buffered root incubations. Production of CH₄ was markedly increased in the marble-buffered incubations. However, one of the phosphate-buffered incubations (no. 19) eventually also exhibited accelerated CH₄ production, as occasionally observed before (21). Two phases of CH₄ production could be distinguished. During the initial phase, CH₄ accumulated to partial pressures of about 0.2 to 0.5 kPa, the values of δ¹³CH₄ decreased to about −80‰ (Fig. 3A), and α increased to >1.07 (Fig. 3B). Such a relatively high fractionation factor typically indicates a strong contribution of CO₂-dependent methanogenesis to total CH₄ production (1, 3, 31, 32, 35). During the second phase, CH₄ accumulated further, δ¹³CH₄ increased again and the fractionation factor decreased, indicating an increased contribution of acetotrophic methanogenesis to total CH₄ production (Fig. 3). The extent to which α decreased (and δ¹³CH₄ increased) was dependent on how much CH₄ eventually accumulated, i.e., much lower values in the marble-buffered incubations than in the phosphate-buffered incubations.

In summary, these results demonstrate that phosphate at concentrations of ≥20 mM inhibited CH₄ production on excised washed rice roots, in particular, by impeding acetotrophic methanogenesis. In phosphate-buffered incubations, only relatively small amounts of CH₄ were produced by reduction of CO₂. Simultaneously, large amounts of acetate (>10 mM) were produced, about half by reduction of CO₂. However, the produced acetate was not further metabolized. In the absence of phosphate, on the other hand, the produced acetate was eventually further metabolized and CH₄ production accelerated.

Molecular analysis has indicated that *Methanosarcina* species are responsible for the observed conversion of acetate to CH₄ on rice roots (16, 21). *Methanosarcina* species are known to exhibit rather high *Kₘ* and threshold values for acetate (19). This may be the reason why acetotrophic methanogenesis started not immediately at the beginning of incubation but only when sufficient acetate had accumulated. Radiolabeling studies have shown that acetate is an intermediate in the conversion of photosynthetically assimilated CO₂ into CH₄ (10). Acetate may be excreted from the rice roots or may be produced by fermentation of other substrates (e.g., sugars or polysaccharides) which may originate from either root exudates or root decay. Fermentative acetate production includes homoacetogenesis from H₂-CO₂ (7), probably by *Sporomusa* species that were shown to be members of the rice root microflora (24). The concentrations of acetate on the rice roots may be relatively sensitive to high phosphate concentrations. Roots excrete phosphatases and dissolve calcium phosphates on roots of wetland plants (29). For phosphate mobilization, roots excrete phosphatasess and dissolve calcium phosphates by acidification (32). Phosphate concentrations inhibit methanogens, acetotrophic ones in particular, is unknown. However, there seems to be a widespread awareness that buffering of culture media with more than 30 mM phosphate may result in inhibition of microbial growth (34), although details and specific observations are rarely described (2, 6). On the other hand, media buffered with 20 to 50 mM phosphate have frequently been used for cultivation of *Methanosarcina* (20, 28). However, the methanogens in the rhizosphere may be more sensitive to phosphate since it will be limiting unless the soil is fertilized. Rice roots efficiently take up phosphate for plant nutrition. Phosphate is only sparingly soluble in the presence of calcium and iron, which are present in paddy soil. Iron phosphates form precipitates on roots of wetland plants (29). For phosphate mobilization, roots excrete phosphatasess and dissolve calcium phosphates by acidification (32). Phosphate concentrations in the rhizosphere of various plants were found to be below 30 to 35 µM (12). Consequently, the methanogenic microflora on plant roots may have adapted to low-phosphate conditions and thus may be relatively sensitive to high phosphate concentrations.

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Our results suggest that the phosphate concentration in the rhizosphere is another factor which possibly regulates acetate turnover and CH₄ production on rice roots. Indeed, it has recently been shown that a low phosphate supply to rice plants results in enhancement of CH₄ emission (22). This response may be due to the release of inhibition of acetotrophic methanogens on the roots. The mechanism by which increased phosphate concentrations inhibit methanogens, acetotrophic ones in particular, is unknown. However, there seems to be a widespread awareness that buffering of culture media with more than 30 mM phosphate may result in inhibition of microbial growth (34), although details and specific observations are rarely described (2, 6). On the other hand, media buffered with 20 to 50 mM phosphate have frequently been used for cultivation of *Methanosarcina* (20, 28). However, the methanogens in the rhizosphere may be more sensitive to phosphate since it will be limiting unless the soil is fertilized. Rice roots efficiently take up phosphate for plant nutrition. Phosphate is only sparingly soluble in the presence of calcium and iron, which are present in paddy soil. Iron phosphates form precipitates on roots of wetland plants (29). For phosphate mobilization, roots excrete phosphatasess and dissolve calcium phosphates by acidification (32). Phosphate concentrations in the rhizosphere of various plants were found to be below 30 to 35 µM (12). Consequently, the methanogenic microflora on plant roots may have adapted to low-phosphate conditions and thus may be relatively sensitive to high phosphate concentrations.

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