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Evidence and quantification of thiosulfate reducers unable to reduce sulfate in ricefield soils

Sylvie Escoffier, Bernard Ollivier, Jean Le Mer, Joël Garcin, Pierre Roger*

Laboratoire Orstom de microbiologie des anaérobies, Université de Provence - CESB/ESIL, Case 925, 163, avenue de Luminy, 13288 Marseille cedex 9, France.

** Corresponding author (fax: +33 4 9182 8570; e-mail: rogerpa@orstom.esil.univ-mrs.fr)*

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Abstract – We provide the first evidence that thiosulfate reduction can be performed in soil under anaerobic conditions by non-sulfate-reducing bacteria. Culturable thiosulfate-reducing bacteria were enumerated in five ricefield soils by MPN counts, using peptides and H_2 as energy sources, and thiosulfate as the electron acceptor. Such conditions favoured the growth of (i) thiosulfate-reducing bacteria unable to use sulfate as electron acceptor and (ii) sulfate-reducing bacteria that did not use lactate as an energy source. Thiosulfate-reducing bacteria ranged from 40 to $4 \cdot 10^4$ cells·g^{-1} dry soil and their abundance was of the same order of magnitude as that of sulfate reducers using lactate. Most probably, thiosulfate-reducing bacteria unable to use sulfate are as important as sulfate reducers for sulfur cycling in some wetland soils. © Elsevier, Paris

Rice / soil / S cycle / sulfate / thiosulfate / microflora / MPN counts

Résumé – Démonstration et quantification de thiosulfato-réducteurs ne pouvant réduire le sulfate dans les sols de rizière. Nous démontrons, pour la première fois, l'existence d'une activité thiosulfato-réductrice anaérobie due à des micro-organismes non sulfato-réducteurs dans le sol. Les populations thiosulfato-réductrices de cinq sols de rizière ont été comptées par MPN sur un milieu contenant des peptides et de l'H_2 comme sources d'énergie et du thiosulfate comme accepteur d'électrons. Ce milieu s'est révélé sélectif pour (i) les bactéries thiosulfato-réductrices non sulfato-réductrices et (ii) les bactéries sulfato-réductrices incapables d'utiliser le lactate comme source d'énergie. La densité des populations thiosulfato-réductrices varie de 40 à $4 \cdot 10^4$ bactéries·g^{-1} sol sec. Ces valeurs sont du même ordre de grandeur que celles des populations de sulfato-réducteurs utilisant le lactate. L'implication des bactéries thiosulfato-réductrices non sulfato-réductrices dans le cycle du soufre de certains sols submergés est probablement aussi important que celle des bactéries sulfato-réductrices. © Elsevier, Paris

Riz / sol / cycle du soufre / sulfate / thiosulfate / microflore / MPN

1. INTRODUCTION

In wetland ricefields, flooding creates anaerobic conditions a few millimeters beneath the soil surface and leads to the differentiation of environments differing in their physical, chemical, and trophic properties. Oxic environments include the floodwater, the submerged parts of the rice plant and the surface soil. Reduced environments include the bulk of the soil and the plow layer. A very large area of the aerobic-anaerobic interface develops near the soil surface and in the rice rhizosphere. As a result, a broad range of redox potential is observed in flooded ricefields, with positive values in the floodwater and surface soil, and values that decrease after submersion up to –200 mV in the bulk of the soil and the reduced zone of the rhizosphere [17]. This has implications for the microorganisms present and the oxidation status of a number of key elements, especially Fe, Mn and S.

In wetland rice soils, sulfur exists at oxidation levels ranging from +6 (sulfate) to –2 (sulfide). At the aerobic-anaerobic interfaces, the pools of oxidized and reduced forms of sulfur can be rapidly interconverted by chemical and microbial reactions [7]. Recently,

Wind and Conrad [25] provided evidence of a very dynamic cycling of both reduced and oxidized forms of sulfur in planted rice soils. They measured (i) concentrations of sulfate and thiosulfate higher than 300 and 150 µM, respectively, in the upper layer of the rice soil and (ii) activities (sulfate and thiosulfate reduction) that were two- to five-fold higher in planted soil than in the unplanted control.

In rice soils, S cycle has two major aspects: plant nutrition and sulfide toxicity to the plant. Rice growth requires an adequate supply of S; depending on variety and yield, a rice crop removes between 8 and 17 kg $S \cdot ha^{-1}$ from the soil [7]. Various toxicities are due to sulfide produced by anaerobic bacteria utilizing oxidized forms of S (sulfate and thiosulfate). Such populations develop at aerobic-anaerobic interfaces, namely the rice spermosphere and rhizosphere where they affect seed germination and plant growth. Hydrogen sulfide has been reported as the causal factor in 12 of the 27 physiological disorders of rice [24]. Hydrogen sulfide may inhibit seed germination [10] or cause the early death of 30–100 % of the crop [11]. Up to now, sulfide toxicity in ricefields had been exclusively attributed to the activity of sulfate-reducing bacteria, which can reduce both sulfate and thiosulfate. However, recent studies have shown the existence of populations of anaerobic thiosulfate reducers unable to reduce sulfate.

Thiosulfate reduction by non-sulfate reducers appears to be quite widespread within the thermophilic and hyperthermophilic anaerobic *Archaea* and *Bacteria* [5, 6, 14, 18–20, 23]. It was also reported for non-thermophilic moderate halophilic microorganisms isolated from oil wells belonging to the genera *Haloanaerobium*, *Spirochaeta* and *Dethiosulfovibrio* [15, 16, 21] suggesting that this metabolism could be more widespread amongst anaerobes than previously thought.

In contrast to sulfate-reducing bacteria, which often require acetate as a carbon source for growth, anaerobic thiosulfate reducers require complex organic compounds, such as yeast extract and peptides, and can oxidize H_2 [5, 16].

In an attempt to provide evidence that, under anaerobic conditions, thiosulfate reduction in ricefields could also be performed by microorganisms other than sulfate reducers, we developed a culture medium and a method for MPN (most probable number) counts of thiosulfate reducers unable to reduce sulfate. The method was applied to five rice soils.

2. MATERIALS AND METHODS

2.1. Ricefield soils

The five soils used originated from ricefields from Camargue (south of France) and the Philippines (Pili, Maahas, Maahas alkaline and peat soil). Their properties were described by Joulian et al. [12]. These soils were chosen to provide a broad range of physicochemical properties: Camargue soil is a moderately alkaline silt fine clay soil; Pili is an acid-sulfate soil; Maahas is a neutral clay soil; Maahas alkaline is a Maahas soil artificially alkalinized in situ by the addition of carbonate; peat is an organic soil (organic C: 15.7 %; total N: 1.5 %) very rich in available P (102 ppm).

2.2. Culture media

Media were derived from the basal medium of Ravot et al. [18] to which various substrates and electron acceptors were added. The basal medium (BM) composition was: NH_4Cl 1 g; K_2HPO_4 0.3 g; KH_2PO_4 0.3 g; $MgCl_2.6H_2O$ 0.1 g; $CaCl_2.2H_2O$ 0.1 g; NaCl 1 g; KCl 0.1 g; yeast extract 0.01 g; cysteine HCl 0.5 g or none as further indicated; resazurin 1 mL; Balch oligoelement solution 10 mL [1]; distilled water 1 L. pH was adjusted to 7.0 with a 10-N KOH solution. Media were distributed in Hungate's tubes (4.5 mL per tube) that were flushed with N_2/CO_2 (80/20; v/v) to ensure anaerobiosis [13].

After autoclaving (45 min at 110 °C), 0.08 % (w/v) Na_2S and 0.5 % (w/v) carbonate buffer were added to the tubes from sterile solutions.

In the basal medium, cysteine and Na_2S were used as reducing agent and resazurin was used as redox indicator, turning pink under inadequate oxidized conditions.

The media used for MPN counts were:

– M1: (BM + Biotrypcase 5 $g \cdot L^{-1}$ + thiosulfate 20 mM + H_2 2 bars in the incubation atmosphere) used to enumerate thiosulfate reducers;

– M2: (M1 without thiosulfate) used as control for M1;

– M3: (BM + sulfate 20 mM + lactate 20 mM) used to enumerate sulfate reducers;

– M4: (M3 without sulfate) used as control for M3.

In addition, we used a M5 medium (M1 with thiosulfate replaced by sulfate 20 mM) to test for the presence of hydrogenotrophic sulfate-reducing bacteria.

2.3. Counts by the MPN method

The 10^{-1} soil suspension-dilutions were prepared by stirring 10 g dry soil and 90 mL anaerobic sterile physiological water (9 $g \cdot L^{-1}$ NaCl) for 1 h. Counts were performed in triplicate by preparing three series of dilutions from three soil subsamples. Half a millilitre of each dilution was inoculated in triplicate Hungate's tubes containing 4.5 mL medium. The growth of thiosulfate and sulfate reducers in the tubes was assessed through sulfide production in the tubes. The composition of the media allowed a similar potential

sulfide production for both sulfate or thiosulfate reduction (20 mM). Sulfide was measured colorimetrically according to Cord-Ruwisch [3] after a one-month incubation, a duration determined from a preliminary two-months experiment.

3. RESULTS AND DISCUSSION

3.1. Identification of MPN positive tubes

The media used contained sulfur compounds other than thiosulfate or sulfate. These were cysteine and Na_2S, used to maintain reducing conditions in the tubes, and Biotrypcase as a C source. Possibly, unknown forms of sulfur were also brought in the MPN tubes by the inoculated soil dilutions. As a result, we always noticed the production of sulfide in control tubes. The first step was therefore to define the threshold beyond which a tube could be considered positive, that is, when an amount of sulfide significantly higher than that in the control was produced. Sulfide produced in control tubes could originate from (i) non-biological reactions, (ii) microbial fermentation of sulfur-containing organic substrates originating from the medium or the soil dilutions, and (iii) the utilization of sulfate and thiosulfate originating from soil dilutions.

The maximum chemical production of sulfide from sulfur compounds present in the medium (Na_2S, cysteine, Biotrypcase, and thiosulfate or sulfate), measured in the non-inoculated control tubes (M1 and M3), was 1.5 mM.

In a first study with the Camargue and peat soils, the average sulfide production in the cysteine containing controls was significantly higher (Student's *t*-test) than those recorded in the non-inoculated controls, indicating that microbial fermentation of S-containing cysteine was responsible for this production. In further enumerations performed on Maahas, Maahas alkaline and Pili soils, we used a medium without cysteine as we observed that Na_2S was sufficient to maintain an adequate redox potential in the tubes.

Threshold values for MPN counts were determined in inoculated controls without electron acceptors (sulfate or thiosulfate).

In cysteine-containing controls, the average sulfide production was 2.4 mM for the thiosulfate-reduction controls and 2.7 mM for the sulfate-reduction controls. The histogram of both sets of data exhibited a normal distribution (*figure 1*). The mean of both sets of data were significantly different ($\varepsilon = 2.18 > 1.96$, $P = 0.05$; $n = 157$). Therefore, we determined two thresholds corresponding to a value higher than 97.5 % of the data (m + 1.96 s). Values were 4.4 mM for thiosulfate-reduction and 5.2 mM for sulfate-reduction.

In controls without cysteine, the histogram of the data exhibited a strongly dissymetrical distribution (*figure 1*). The study of the correlation between mean and variances of replicated sets of measurements

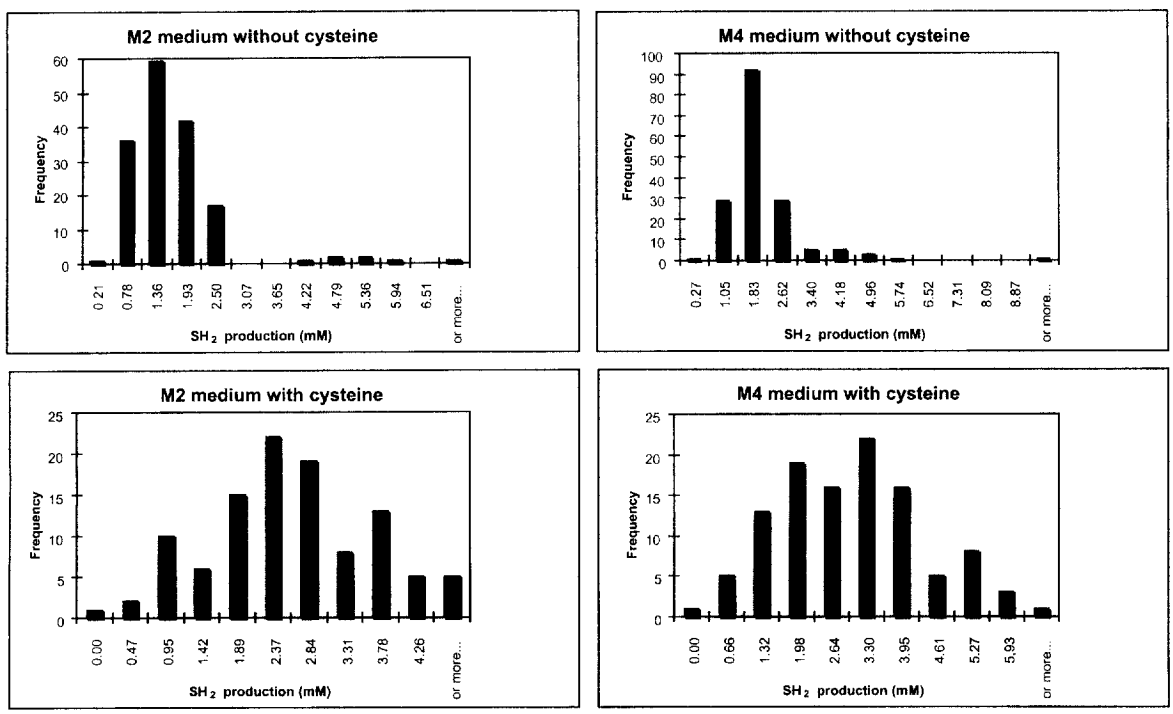

Figure 1. Histograms of sulfide concentrations recorded in control tubes (M2 and M4) with or without cysteine.

Table I. MPN counts of thiosulfate-reducers on M1 medium and sulfate-reducers on M3 medium in five dry ricefield soils.

Soil			Thiosulfate-reducers		Sulfate-reducers	
Name	Type	pH	nb·g^{-1} dry soil*	C.V.** (%)	nb·g^{-1} dry soil*	C.V.** (%)
Camargue	Alkaline silt/fine clay soil	7.7	$6.2 \cdot 10^3$	5	$2.5 \cdot 10^4$	0
Pili	Acid-sulfate soil	3.9	$1.4 \cdot 10^2$	12	$1.1 \cdot 10^2$	88
Maahas	Neutral clay soil	5.9	$1.1 \cdot 10^3$	10	$6.7 \cdot 10^3$	7
Maahas alkaline	= Maahas + carbonate in situ	7.9	$3.7 \cdot 10^4$	3	$1.2 \cdot 10^4$	9
Peat soil	Organic soil rich in available P	6.4	40	87	$1.9 \cdot 10^2$	15

*, Average of counts on three soil samples; **, coefficient of variation (%).

showed a significant correlation with a slope for the regression curve (b) of 1.93 for thiosulfate-reduction (n = 12) and 1.97 for sulfate-reduction (n = 10) indicative of a log-normal distribution [22]. The comparison of the data normalized by $y = x^{(1 - b/2)}$ showed that the means of both sets of data were not significantly different ($\varepsilon = 0.66 < 1.96$; $P = 0.05$; $n = 157$). A single threshold value of 4 mM of sulfide was calculated for the thiosulfate-reduction and the sulfate-reduction controls using the logarithm of the data according to the method described by Roger et al. [22].

The difference observed in the distribution law of the sulfide production in the two types of controls may translate the nature of the microflora involved. Microbial activities are often distributed according to a log-normal distribution [22]. However, activities resulting from a broad range of organisms may follow a normal distribution, as observed for denitrifying bacteria [8]. Sulfide production in inoculated controls without cysteine, corresponded mostly to the activity of thiosulfate or/and sulfate reducers using the substrates brought by the soil, which led to a log-normal distribution. Sulfide production in inoculated controls with cysteine corresponded to the activity of a broad range of organisms including fermentative bacteria, and thiosulfate or/and sulfate reducers, which led to a normal distribution.

3.2. Counts

MPN counts on sulfate ranged from 10^2 to $2 \cdot 10^4$ g^{-1} dry soil. These values are of the same order of magnitude than those reported by Garcia et al. [9] in 29 ricefield soils in Senegal. MPN counts on thiosulfate ranged from 40 to $4 \cdot 10^4$; no value was available for comparison.

Counts on sulfate and thiosulfate usually exhibited a satisfactory reproducibility, with coefficients of variation lower than 15 % in eight of the ten cases (*table I*). However, bacteria counted on thiosulfate (TSRB) in peat soil and sulfate-reducing bacteria (SRB) counted in Pili soil exhibited high coefficients of variation of up to 88 %. Both soils had very low populations and the variability of the data partly resulted from the difficulty of performing colorimetric measurements in low dilution tubes (10^{-2}), containing a relatively dense soil dilution.

The study of the correlation between means and variances of replicated counts showed a log-normal distribution for both TSRB (b = 1.67) and SRB (b = 1.88). Therefore, correlations between soil properties and population abundance were studied by using the logarithms of the population numbers. We observed a weak correlation between the abundance of TSRB and soil pH ($r = 0.76$; $P = 0.1$) and a significant correlation between SRB and soil pH (r = 0.85; $P = 0.05$). The highest populations of TSRB were found in alkaline Maahas soil (pH = 7.9) whereas the highest populations of SRB were found in Camargue soil (pH = 7.7). Despite a high content in sulfate, acidic Pili soil (pH = 3.9) exhibited low populations of TSRB and SRB. No significant correlation was found between TSRB et SRB suggesting the existence of two distinct populations.

3.3. Occurrence of thiosulfate reducers unable to reduce sulfate in soils

Cross-inoculation experiments to determine the occurrence of thiosulfate reducers unable to reduce sulfate were conducted with Camargue and Maahas soils where a significant density of thiosulfate reducers was recorded.

Thiosulfate reducers growing in MPN tubes containing M1 medium were checked for sulfate reduction by inoculating tubes containing M3 medium with 0.5 mL M1 positive dilutions (10^{-2}–10^{-4}). Simultaneously, the viability of the inocula was tested by reinoculating them in M1 medium. The results are summarized as follows:

– all M1 controls exhibited a positive growth;

– a production of sulfide higher than the threshold (4 mM) was observed in only 38 % of the M3 tubes inoculated with positive M1 dilution, and it remained low (< 7 mM);

– 62 % of the M3 tubes did not produce sulfide;

– all M3 tubes were negative when inoculated with the corresponding M1 10^{-4} dilution.

These results demonstrated:

i) the presence of thiosulfate-reducing bacteria unable to reduce sulfate with lactate as the electron donor;

ii) that M1 medium (thiosulfate + Biotrypcase + H_2, 2 bars) was selective for thiosulfate reducers unable to utilize sulfate, but also possibly allowed the isolation of hydrogenotrophic SRB unable to oxidize lactate.

To test the last hypothesis, we inoculated the last positive dilution tubes of thiosulfate-reducing enrichments from Camargue and Maahas soils in a modified M1 medium where thiosulfate was replaced by sulfate (M5). Enrichments from Camargue soil could also oxidize H_2 in the presence of sulfate whereas those from Maahas soil did not. Results with Maahas soil confirmed that medium M1 (peptone + thiosulfate + H_2) was selective for thiosulfate reducers unable to reduce sulfate. Results with Camargue soil showed that sulfate-reducing bacteria unable to use lactate were also recovered on M1 medium. Microscopic examinations of the Camargue soil enrichments showed the absence of vibrio, indicating that enriched microorganisms did not belong to the genus *Desulfovibrio* usually dominant on lactate.

4. CONCLUSION

This work aimed at elucidating if thiosulfate reduction could be performed in ricefield soils, under anaerobic conditions, by non-sulfate-reducing bacteria.

The composition of the medium used to estimate populations of thiosulfate reducers took into account the recent observations that (i) most anaerobic thiosulfate-reducing bacteria require organic complex compounds such as peptides for growth and (ii) they oxidize H_2. Results showed that MPN counts of thiosulfate-reducing bacteria can be achieved by using peptides and H_2 as energy sources and thiosulfate as the electron acceptor on a medium devoid of cysteine; Na_2S was sufficient for maintaining a low redox potential. Such conditions selected thiosulfate-reducing bacteria unable to use sulfate as electron acceptor in Maahas soil, but also sulfate-reducing bacteria unable to oxidize lactate in Camargue soil.

In the five studied soils, representing a broad range of physico-chemical properties, populations of sulfate-reducing bacteria estimated by MPN (using lactate as the energy source and sulfate as the electron acceptor) ranged from $1.1 \cdot 10^2$ to $2.5 \cdot 10^4$ cells$\cdot g^{-1}$ dry soil. Populations of thiosulfate-reducing bacteria were of the same order of magnitude, ranging from 40 to $3.7 \cdot 10^4$ cells$\cdot g^{-1}$ dry soil. No significant correlation was found between both populations.

Our results provide the first evidence of the presence of anaerobic thiosulfate reducers that are not sulfate reducers in ricefield soils and in soil in general. The characterization of the isolated strains will be published elsewhere. Our results also indicate that the enumeration of SRB in soils, using only lactate as the substrate (as most often practised), may underestimate populations of SRB because it does not take into account SRB using H_2.

The presence of thiosulfate reducers unable to reduce sulfate in ricefields may partly explain the high thiosulfate consumption potential reported by Wind and Conrad [25] in rice soils. With regard to the densities recorded in some soils, such microorganisms may contribute significantly to the production of H_2S at the aerobic/anaerobic interfaces, where thiosulfate is available, especially in the rhizosphere. Therefore, in a number of rice soils, thiosulfate reducers unable to reduce sulfate are probably as important as sulfate reducers in causing sulfide toxicities to the plant. Because thiosulfate non-sulfate-reducing bacteria can consume H_2 [5, 20], they might also affect N and C cycles. In particular, the effects of the use of (i) ammonium sulfate as N fertilizer to reduce CH_4 emissions from ricefields [4] and (ii) gypsum as an amendment for sodic and/or alkaline rice soils [2] must have impacts on sulfur cycling populations that deserve to be examined, taking into account the occurrence of thiosulfate reducers unable to use sulfate.

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