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A simple membrane-filter technique for the enumeration of S-reducing bacteria in soil and water samples

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Summary

A new membrane-filter technique, suitable for field-surveys, was developed to quantify S-reducing bacteria in waters and soil or sediment samples. Samples, diluted if necessary, were passed through S-coated filters, previously coated with a $Fe(OH)_2$ film. These filters were incubated in screw-cap test-tubes containing a selective medium, in which sodium acetate was the sole electron source. Blackening of the filter, resulting from iron monosulfide production was used as internal growth indicator; and the times necessary for total blackening of each filter to occur were correlated to the initial numbers of viable cells deposited on it.

Key words: Enumeration; Membrane-filter; Sediment; Soil; S-reducing bacterium; Water

Introduction

The S cycle is one of the most important biological cycles in natural environments. Many groups of bacteria are involved in the turnover of reduced and oxidized inorganic S compounds in anaerobic soils or anoxic waters. The isolation and characterization data on *Desulfuromonas acetoxidans* [1] have shown that production of sulfides may occur as the result of a microbiological process which differs from the well-known dissimilatory sulfate reduction. This genus of strictly anaerobic bacteria is able to significantly reduce elemental S to hydrogen sulfide, using acetate, ethanol and propanol- or butanol-succinate mixtures as electron donors. This strain differs from some peculiar sulfate-reducing bacteria, all belonging to the genus *Desulfovibrio* [2], with which S reduction is an alternative process, but not occurring at a significant rate, however.

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In tropical areas mainly, sulfides in anaerobic soil solution induce toxic conditions which are unfavourable to some crops cultivated in water-logged soils [3–5]. It is, therefore, worth enumerating not only the sulfate-reducing bacteria, especially the non-sporulating forms (*Desulfovovibrio*) evidenced as major sulfide producers [5], but also the S-reducing bacteria which may also play a role in production and accumulation of significant amounts of gaseous or soluble sulfides in ricefields. A double survey of this kind would be of great economic value to some developing countries, as well as Japan [6] or the southern USA [7].

Many different techniques are available for bacterial enumerations in samples from various natural environments: immunofluorescence [8–10], ATP determination [11], use of radioactive substrata [12, 13] or the very frequently used counting plates methods [14, 15]. Because most of them require sophisticated laboratory equipment, they are not easily adaptable to field-use. The technique presented in this paper is quite similar to those used to enumerate autotrophic thiobacilli [16], and has similar potential for use during field-surveys to process samples, which need only to be filtered.

Materials and Methods

Specific medium

Growth experiments on S-reducing bacteria were carried out at 30 °C in 22-ml Kimax screw-cap test-tubes, filled with the medium freshly prepared from the following two stock solutions.

Solution A. KH_2PO_4, 1 g; NH_4Cl, 0.3 g; $MgCl_2 \cdot 7H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.3 g; NaCl, 20 g (for saline-tolerant strains only); modified Pfennig's element trace solution [17], 10 ml; and distilled water, 900 ml. This solution was sterilized by autoclaving at 110 °C for 30 min.

Solution B. $NaHCO_3$, 2 g; 0.4% (w/v) biotin solution, 1 ml; 0.02% (w/v) vitamin B_{12} solution, 1 ml; 5% (w/v) $CH_3COOHNa$ solution, 10 ml; and distilled water, 100 ml. This solution was sterilized by microfiltration.

1 l specific medium was obtained by mixing Solutions A and B. Final pH was ≈7.2 and, if necessary, was adjusted to this value, using 1 N NaOH. This solution was aseptically distributed among 22-ml screw-cap sterile test-tubes containing 50 mg finely ground FeS and 1 ml distilled water.

Preparation of S-coated filters

The S-coated filters were obtained using the technique previously described by Mouraret and Baldensperger [16]. In a 1.5-l flask, a colloidal S solution was prepared by mixing 2 ml concentrated HCl and 100 ml 10% (w/v) $Na_2S_2O_3$ solution. After 10 min, 1 l demineralized water was added. The resulting mixture allowed to set for 20 min. Then, 150 ml of the suspension were filtered through edge-hydrophobic filters (Millipore HAEG 047 A0), using Sterifil filter holders (Millipore XX 11 4700). In each filter, S was deposited by ≈40 mg S granules, 0.3–30 μm in diameter [18]. They were immediately washed by passing through 1 l sterilized, demineralized water and allowed to dry. Storage of S-coated filters before use, in a dry and sterile place,

is possible for ≈1 wk without significant modification of their properties.

Processing of samples and inoculation methods

Liquid sample processing. Just before use, S-coated filters were dipped in 1% (v/v) Tween 80 solution for at least 5 min and placed on the same Sterifil apparatus. Then, 100–500 ml of liquid samples were passed directly through filters, using a manifold (Millipore XX 2604735), in which a vacuum is created by the means of a manual pump (Arthur Thomas 1015). Water samples were run at least in triplicate.

Soil and sediment sample processing. Immediately after collection, 5 g reduced soil or sediment was ground in a mortar and suspended in sterile and anoxic 0.5% (w/v) $MgCl_2 \cdot 7H_2O$ solution to a final volume of 100 ml. A 2.5-ml aliquot was diluted to a volume of 250 ml in freshly sterilized water to obtain Dilution 1. A 20-ml aliquot of this solution was likewise diluted to a volume of 250 ml to obtain Dilution 2. Then, 50 ml Dilution 1 and 250 ml Dilution 2 were, respectively, filtered on a S-coated filter, as previously described (see previous section).

These two filters, therefore, received, respectively, 1/40 and 1/200 bacteria of the whole sample. Each filter containing bacteria was immediately covered (as soon as possible, to ensure reductive conditions on its surface) with a deposit of $Fe(OH)_2$, which was prepared as follows (Solution C):

Solution C_1: $FeCl_2 \cdot 4H_2O$ (2 g) + distilled and sterilized water (50 ml);

Solution C_2: $NaHCO_3$ (2 g) + distilled and sterilized water (50 ml).

Solution C is a mixture of Solutions C_1 and C_2 diluted to a volume of 1 l, corresponding to the $Fe(OH)_2$ stoichiometry:

$$FeCl_2 \cdot 4H_2O + NaHCO_3 \rightarrow \textit{Fe(OH)}_2 + 2\,NaCl + CO_2 + 4H_2O$$

10 ml Solution C was passed through each filter and the resulting deposit corresponded to ≈5 mg Fe(II). Finally, the inoculated membranes were very rapidly introduced into test-tubes filled with the growth-specific medium and immediately incubated at 30 °C. The duration of incubation was measured from this time.

The total time between the sampling and the introduction of the filter in the tube ought to be as short as possible to avoid the death of anaerobic cells during air exposure. In aerated liquid cultures, it was observed that most cells of strains used during this study were killed in ≈10 min but also that in reduced soil solutions, they were quite well protected, mainly in soils high in clays.

Principle of quantification of samples and inoculation methods

The reaction time (expressed in hours and days) was needed to obtain a very dark deposit on the two faces of the membrane. The blackening of the filter, easily observed in glass tubes (Fig. 1), was used to characterize the positive growth of the initial S-reducing bacterial population. With a water-logged sample, the number of bacteria was expressed in gram of dry soil or sediment and corrected depending on the moisture level of the sample.

The black deposit in the filter is due only to iron monosulfide (FeS), formed when H_2S, resulting from S reduction, reacts with $Fe(OH)_2$:

$$CH_3COOH + 2\,H_2O + 4S^0 \rightarrow 4\,H_2S + 2\,CO_2 \quad \text{(biotic)}$$

$$4\,Fe(OH)_2 + 4\,H_2S \rightarrow 4\,FeS + 8\,H_2O \quad \text{(abiotic)}$$

$$CH_3COOH + 4S^0 + 4\,Fe(OH)_2 \rightarrow \textit{4 FeS} + 2\,CO_2 + 6\,H_2O$$

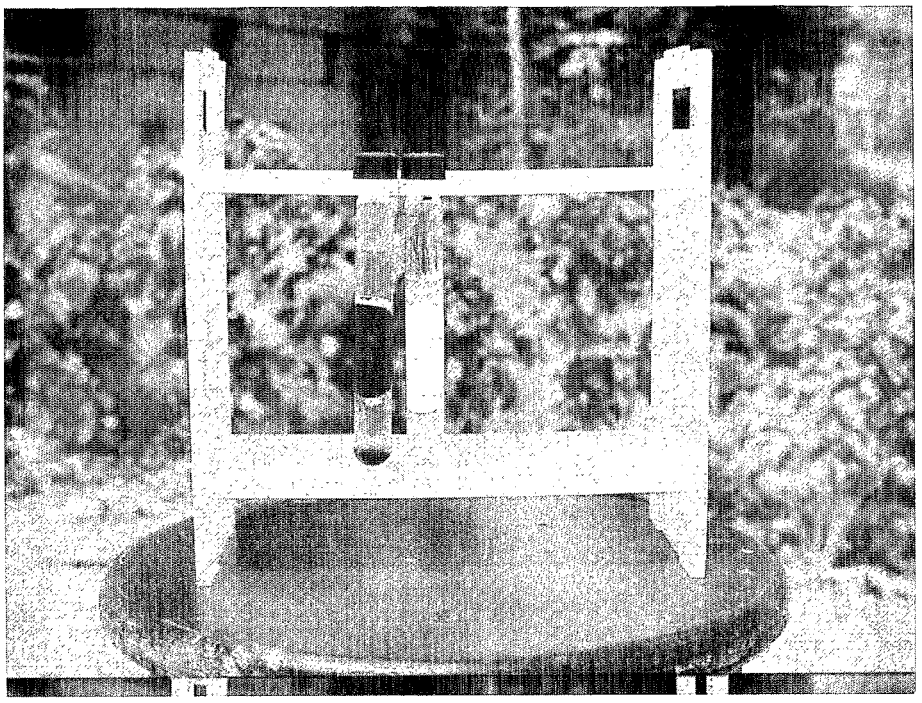

Fig. 1. Inoculated tubes: after (right) and before blackening (left) of S-coated filter.

Calibration curve

Three S-reducing strains isolated from tropical lowland rice soils were used to correlate the time necessary to give a positive growth with the initial numbers of viable cells deposited on the filters. These isolates represented three different ecological niches and climates in coastal West Africa: PG (semidesert Senegal delta), NIA (tropical mangrove rainy zone) and DJ (nonsaline water-logged rice soil in the Casamance estuary). Cell numbers in initial culture medium were determined by microscopic observations. Serial dilutions were then prepared anaerobically in sterilized 0.5% (w/v) $MgCl_2$ solutions, in a total volume of 100 ml; a 50-ml aliquot of each dilution was supplemented with 50 mg of the corresponding sterilized soil and passed through a S-coated filter. Membranes thus prepared were used as inoculum. Each serial dilution sample was run in triplicate. In some extra duplicates, a resazurin solution ($1 \cdot 10^{-6}$ in final concentration) was added to verify that anaerobiosis remained convenient in tubes when they were opened to insert the filter. So, it was verified that the typical pink color (at pH 7.2) resulting from significant reoxidation did not appear.

Since the number of cells deposited on each filter was known, standardized time course curves could be easily obtained. They were quite similar for the three strains (Fig. 2), in spite of a difference in salinity of their biotope. Thereafter, these results were used to establish a calibration curve, useful for calculating the average number of bacteria $\cdot g^{-1}$ of samples from unknown soils of the same geographical area.

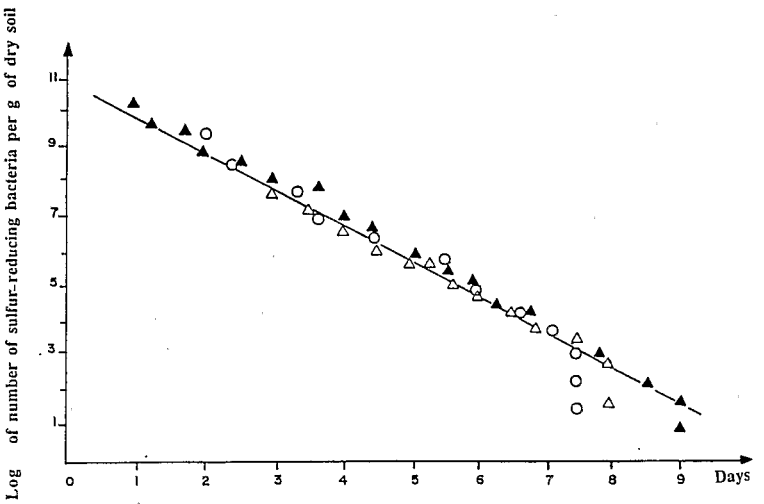

Fig. 2. Calibration curves for three S-reducing bacteria isolated in Senegal: ○, Pont-Gendarme (PG) strain; ▲, Niambalang (NIA) strain; △, Djibelor (DJ) strain.

Results and Discussion

Fig. 2 shows the standard curve obtained by correlating the log of initial numbers of S-reducing bacteria deposited on the filter with the corresponding incubation times, whatever soil or isolate. As shown, a straight line results providing the growth medium is appropriate. The relationship between the log of initial cell number (y) and the produced sulfide (x) obeys the general equation $y = ax+b$. The slope of the curve is always negative (the log decreases as the incubation time increases) and depends on the temperature. It is, therefore, necessary to place the tubes at a chosen constant temperature quite close to the optimal temperature of the strain or/and those of the sampling ecosystem. The curve in Fig. 2 was obtained at 30 °C. As far as the dilution technique is concerned, the actual number of cells $\cdot g^{-1}$ soil or $\cdot ml^{-1}$ culture was assessed with a good degree of accuracy. The relationship between the number of cells and the incubation time was accurate as long as this cells number was $\geq 20-30 \cdot \text{filter}^{-1}$.

Any previous technique was elaborated to enumerate S-reducing bacteria, not only for field-use, but even for laboratory-use in typical strain of *D. acetoxidans*. Any misleading may be due to the sensitivity of cells to aeration during both sampling and filtration. It was assumed that cells in soil dilutions did not suffer from the O_2 stress more severely than those diluted to establish the calibration curve, providing that the dilution of sample was done in a very short time and that the initial number of cells was sufficiently high. It was necessary to initiate growth with the largest number of living cells in order to minimize the lag phase. But the size of diluted sampling of soil to pass through the filter was limited by clogging of the membrane, either by colloids or by clays: porosity of S-coating is obviously lower than those of bare membranes. Similar difficulties were encountered by Mouraret and Baldensperger to enumerate thiobacilli [16] on soils. This limit did not exist for liquid samples or for enumerations

on isolated strains growing in a synthetic medium.

This new technique has been used to assess the distribution of S-reducing bacteria in some tropical soils and sediments in West Africa. The results of 70 samplings from Senegal, The Gambia and Ivory Coast are given in Table 1. One can see that this trophic group of bacteria is widespread in the tested soils and that the total population depends less on the level of soil hydration than on the physico – chemical structure of the soil. It would be useful to compare these data with those obtained in other countries and from different natural biotopes ... but any similar countings were published elsewhere! Our surveys in tropical areas [5] asserted that the obligate S-reducing bacteria may contribute, but less than sulfate reducers, to the toxic sulfide accumulation in water-logged acidic rice paddy fields containing high amounts of both sulfates and organic matter, even when Sulfur Coated Urea is used as fertilizer.

Mangrove sediments, in which NaCl concentrations are very high, contain a small amount of S-reducing bacteria. However, paddy rice fields established on reclaimed (and partly desalinized) mangrove soils were found to have high populations. It, therefore, appears that they may be sensitive to the choride concentration, which differs significantly between the true mangrove soils, which are inundated by the tide or brackish water twice a day, and barren or herbaceous tidal zones. But some strains typical from ricefields established on mangrove soils, as NIA isolate used during this work, remained very active on the salted synthetic medium containing 20 g sodium chloride $\cdot l^{-1}$.

TABLE 1

QUANTIFICATION OF S-REDUCING BACTERIA IN SOME TROPICAL SOILS IN WEST AFRICA

Type of soil	Sampling				S-reducing bacteria: mean value on total samples ($\times 10^3$ cells $\cdot g^{-1}$ soil)
	Country	Sites	Dry (D) or rainy (R) season	Number of samples	
Fluvio – marine alluvial soils	Senegal	Pont-Gendarme	D	6	$7.76 \cdot 10^4$
	Senegal	N'Diaye-N'Delle	D	4	$3.64 \cdot 10^6$
Mangrove tidal sediments	Senegal	Basside	D	5	$4.00 \cdot 10^4$
	Senegal	Basside	R	6	$8.10 \cdot 10^3$
	Senegal	Missirah	R	4	$2.00 \cdot 10^2$
	Senegal	N'Dimsirah	R	5	$8.40 \cdot 10^1$
	Senegal	Tobor	R	4	$5.11 \cdot 10^1$
	Senegal	Balingor	R	5	$2.70 \cdot 10^3$
	Gambia	Farafeny	R	5	$4.20 \cdot 10^4$
Cultivated fields	Senegal	Niambalang	R	4	$2.80 \cdot 10^5$
Farmer mangrove soils	Senegal	Ziguinchor	R	6	$3.00 \cdot 10^6$
Alluvial soils	Senegal	Djibèlor	D	4	$2.10 \cdot 10^2$
	Senegal	Djibèlor	R	5	$7.00 \cdot 10^6$
	Ivory Coast	Yabra	R	5	$3.00 \cdot 10^6$
	Ivory Coast	Nieki	R	2	$8.85 \cdot 10^3$

Growth experiments have been performed at 30 °C and pH 7.2 in 16-ml test-tubes.

The use of sulfide changes to enumerate sulfide producing bacteria (such sulfate reducers) was first developed by Spurny et al. [19]. Membrane-filters, on which samples were deposited, were first used by Tilton et al. [20] with marine thiobacilli, and by Tuovinen et al. [21] for *T. ferroxidans* enumerations. A convenient method for field-use was published by Mouraret and Baldensperger [16] for the purpose of autotrophic thiobacilli quantification.

The method described in this paper is the first field-adaptable procedure for use with obligate S-reducing bacteria. Because it is based on hydrogen sulfide production, it was necessary to choose a very selective medium, free from any soluble sulfide and, of course, unsuitable for detection of other sulfide-producing microorganisms. Our medium was adapted from those described by Pfennig and Widdel [1]. The four main differences between the two media may be summarized as follows:

(1) To avoid interactions with acetate oxidizing sulfate-reducing baceria, sulfate was omitted. All the sulfate salts were replaced by other salts.
(2) Although ethanol could have been used instead of acetate as electron donor, we did not use it because some sulfate-reducing bacteria have been reported to reduce elemental S in the presence of ethanol [2].
(3) Good results can be obtained without shaking the inoculated test-tubes during incubation.
(4) Sodium sulfide ($Na_2S \cdot 9H_2O$) was omitted from the growth medium to prevent reactions with the iron hydroxide deposited on the filter, which would have resulted in blackening the filter and liquid medium prior to the bacterial growth.

Other reducing agents were tested to ensure that they sufficiently lowered the redox potential (*Eh*): sodium thioglycolate (0.5%, w/v), ascorbic acid (0.5%, w/v) and solid FeS (50 mg · test tube^{-1}). The best results were obtained when ferrous monosulfide was used, and it was observed that its use did not influence the blackening of filters. It was also microscopically verified that the use of any of these reducing agents did not significantly modify the morphology of observed S-reducing cells of the three tested isolates.

The storage of sterile assay solution during at least 6 months did not result in any chemical blackening of sterile S- and Fe-coated filters, even in the presence of granular FeS.

This technique has been applied to enumerations of sulfate-reducing bacteria [5], replacing the elemental S deposited on the filter by sodium sulfate (20 mM) directly added to Solution A, and choosing an appropriate nonsaline medium (1 g $NaCl \cdot l^{-1}$) also adapted from those of Pfennig and Widdel [1]. The edge-hydrophobic filter may be replaced by a more classical membrane (as Millipore HAWP 047 00). Various electron donors, such as lactate and pyruvate (20 mM), acetate (10 mM), *n*-butyrate (10 mM) and even long-chain fatty acids (as palmitate, at 1 mM), were tested to differentiate *Desulfovibrio* from *Desulfotomaculum* species. It is not necessary for enumerations of sulfate-reducing bacteria to cover the sample deposit on the filter with Solution C of $Fe(OH)_2$, if the medium contains sufficient reduced Fe, as provided not only by granular FeS but also by addition of Mohrs' salt at 6 g · l^{-1} [5]. Biotin may be omitted or replaced by yeast extract (1%, w/v) or by 1 ml Pfennig's vitamins solution [17].

It became possible, by this way, to compare, in the same dilution of the field sample:

(1) the respective levels of S- and sulfate-reducing bacteria; and (2) the populations of all the main bacteria working in biological S cycle when thiobacilli are simultaneously enumerated using the method of Mouraret and Baldensperger [16].

In conclusion, this new technique of cell enumeration has many advantages over the classical cell-counting methods [14, 15]: (1) It is well adapted to field use. (2) It allows easier enumeration of bacteria growing on media containing an electron acceptor so scarcely soluble as elemental S. (3) Its efficiency is very high since its accuracy is $\approx 20-30$ cells $\cdot$ filter^{-1}, i.e., $\approx 20-30$ cells $\cdot 250$ ml^{-1} of liquid sample or of soil dilution.

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