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Characterization of sulfate-reducing bacteria isolated from Senegal ricefields

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1. SUMMARY

Sulfate-reducing bacteria were enumerated in soils and water samples from Senegal ricefields using lactate and sulfate as substrates. When rice plants were severely injured by sulfides, maximum densities ranged from 10^7 to 10^9 bacteria g^{-1} of dry spermosphere or rhizosphere soil. Seven non-sporulating, mesophilic strains were isolated. The strains had motile curved cells and stained Gram-negative. Lactate, pyruvate, $H_2 + CO_2$, malate, fumarate, or ethanol could serve as electron donors. Organic acids were incompletely oxidized to acetate. Alcohols were degraded to the corresponding fatty acids. Sulfate, sulfite, or thiosulfate could serve as electron acceptors and were reduced to sulfide. Vitamins, yeast extract, Biotrypcase, or additional NaCl were not required for growth. On the basis of morphological and physiological properties, and the G + C mol % of the DNA, six isolates were identified as *Desulfovibrio vulgaris* and one as *Desulfovibrio desulfuricans*. The comparison of their main physiological properties with the physico-chemical properties of sampling sites indicated that they were better adapted to conditions prevailing in the rice rhizosphere than to those prevailing in the bulk of soil.

2. INTRODUCTION

Microbial reduction of sulfate is performed by the morphologically and physiologically versatile dissimilatory sulfate-reducing bacteria, an heterogenous group of microorganisms exhibiting a large nutritional diversity [1–3]. As in any natural environment, the activity of sulfate reducers in ricefields depends upon redox potential, sulfate, temperature, salinity, pH, and the availability of organic nutrients provided by rice exudates or derived from the metabolism of heterotrophic fermentative bacteria. Although most rice soils in the world are S-deficient, significant reduction of sulfate has been reported in a wide range of ricefields [4]. Production of sulfides, at levels that cause rice injury, was observed in Asia [5,6], USA

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[7,8], and Senegal [9]. In lowland ricefields, strictly anaerobic bacteria, including sulfate reducers, become active when the soil becomes anoxic, because O_2-consumption by facultative anaerobes is not balanced by O_2-diffusion from the floodwater and the oxidating power of rice roots [10,11].

Sulfate-reducers are common in flooded soils [3,12,13]. They are present in oxidized and reduced soil layers, as colonies located in O_2-depleted sites such as iron-clay aggregates and organic debris [14,15]. They are also found in the rhizosphere and the spermosphere of rice [9,13,16]. Reported densities in rice soils range from 10^3 to 10^5 cells g^{-1} dry soils in Asia [12]. Studies in Senegal ricefields showed the existence of a very active anaerobic microflora, including methanogens [13], sulfate reducers [9,13], sulfur reducers [17], and ferric iron reducers [18] located in the soil and the vicinity of the roots. Pot experiments with seedlings showed densities of sulfate reducers ranging from 6×10^1 to 7×10^5 cells g^{-1} of soil in rhizospheric samples [13], and microplot experiments showed that in situ densities may reach 10^9 cells g^{-1} of soil [16].

Although *Desulfovibrio* strains were suspected to be dominant in Asian ricefields [12], there is only one report on the isolation of both sporulating and non-sporulating sulfate reducers [19]. Simultaneous enumeration of lactate-using sulfate reducers and Thiobacilli [20], suggested that the most numerous sulfate reducers in the spermosphere and the rhizosphere, and, because of their locations, the most potentially harmful for the plant, were mostly non-sporulating forms. This work follows several applied studies of the conditions inducing sulfide toxicity in Senegal ricefields [9,11,16,21]. It reports on the isolation and characterization of seven strains of *Desulfovibrio*. Physiological properties of these strains are discussed with regard to their growth conditions in the sampling sites and the rice rhizosphere.

3. MATERIALS AND METHODS

3.1. Sampling sites

Surveys were done in 82 ricefields located in the delta of the Senegal River and the Casamance, the two rice producing areas of Senegal. In situ soil temperature varied from 28°C to 30°C at 5–10 cm depth under waterlogged conditions [21]. Soil pH ranged from 4.0 to 5.2 in newly rewetted soils and from 5.2 to 6.9 after four weeks of submersion [21].

Four sites with high intensity of microbial reducing processes and marked detrimental effects on rice growth, were chosen for detailed surveys during three rice cropping seasons [11,18,21]. Soils were sampled at time intervals in the root vicinity (rhizosphere) and around germinating seeds (spermosphere). Sites from which the strains were isolated have been described in previous papers [9,16,18,21,22]. Their main characteristics are summarized in Table 1. These soils differed widely in texture and salt content. A lower variability was observed for carbon content and soil pH measured on newly rewetted soils. Senegal River delta soils became less acidic than Casamance soils after 4 weeks of submersion. Despite a low organic matter content, they were favourable to chemical and microbial reducing processes when waterlogged, because of their high clay content [22,23]. Tilène 3 soil was a moderately saline, acidic and sulfated soil [16,23]. Pont-Gendarme 9 soil is highly sulfated and acidic, with a sulfuric horizon and jarosite mottles, and 2‰ w/w mineral sulfur in surface layer [23]. In the Casamance, Djibélor soil, after 30 years of rice cultivation, lost some properties of the original mangrove soil: in soil surface part of soluble sulfates was leached and pH became almost neutral, but jarosite mottles were still encountered under the plough layer [22]. Loudia-Ouoloff soils were acidic mangrove-type soils, in which the anaerobic bacterial process most potentially harmfull to rice was Fe^{2+} production by ferric iron reducers [22].

3.2. Field enumerations

In situ enumerations of sulfate reducers were performed on 3000 soil or water samples (5–10 g) collected in fields exhibiting clear sulfide toxicity symptoms (Fig. 1). Soil samples were collected anoxically at 3–15 cm depth in the spermosphere or the rhizosphere of rice, as described by Prade et al. [22]. Millipore filters were used for the enumeration procedure, as described by

Baldensperger and Mouraret [24] for Thiobacilli, and by Traoré and Jacq [17] for sulfur-reducing bacteria. Samples were diluted in sterile 0.6% $MgCl_2$ solution, homogenized by a rotary shaker, and immediately deposited on Millipore filters (HAWP 04700; Millipore Corp., Bedford, MA; 0.45 μm, 6 filters/sample). Each filter was then placed in a screw-cap tube (22 ml; 125 × 16 mm) containing 21 ml of Medium 1. Tubes were incubated at 35°C until the appearance of a black FeS-precipitate on the filter. The time necessary for total blackening of the filter was correlated with the initial numbers of viable cells [17]. Calibration curves were established with pure cultures of sulfate reducers.

Medium 1 was used for enumeration studies. Solution A: KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $Na_2SO_4 \cdot 10H_2O$, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; modified Pfennig's element trace solution [25], (1 ml); distilled water, 750 ml. Solution B: NH_4Cl, 1.0 g; 60% sodium lactate, 6 ml; $FeSO_4(NH_4)_2 SO_4 \cdot 6H_2O$, 6.0 g; yeast extract (Difco) 1.0 g; distilled water, 235 ml. Solution C: NaOH, 10 ml of 0.43 M solution. Solutions A and C were autoclaved for 20 min at 120°C. Medium 1 was prepared by mixing cooled solutions A and C to B, sterilized through Millipore filters (0.45 μm) and 21 ml of the final medium were aseptically dispensed into sterile Kimax test-tubes containing 50 mg of finely ground FeS and 1 ml of water. The final pH was 7.0. During the last field experiment on Casamance (Loudia-Oualoff) a modification of Medium 1 was tested, in which yeast extract was replaced by 1 ml l^{-1} of 0.4% (w/v) biotin solution and 1 ml l^{-1} 0.02% (w/v) vitamin B_{12} solution [17]. Modified media, with lactate replaced by Na-acetate (10 mM) or Na-palmitate (2 mM), were used to enumerate other types of sulfate-reducing bacteria.

3.3. Enrichment cultures

Enumeration tubes in which calculated numbers of sulfate-reducing bacteria were higher than 10^8 cells g^{-1} of dry soil were kept for enrich-

Table 1

Some isolation site and strain growth characteristics

Site characteristics Sampling zone	Senegal River delta		Casamance	
	Tilène 3	Pont-Gendarme 9	Loudia Oualoff	Djibélor 4
Soil				
Average temperature (°C) [a]	29–31	28–32	28–30	26–28
pH (dry soil) [b]	4.0–4.4	3.8–4.7	4.1–4.4	3.7–5.5
pH (soil solution) [c]	6.1–6.5	5.6–6.2	4.3–6.4	4.2–6.8
SO_4^{2-} $-(10^{-2}$ M·kg$^{-1})$ [b]	3.2–4.3	7.7–9.3	0.73–1.26	0.55–2.2
Na^+ $(10^{-2}$ M·kg$^{-1})$ [b]	~ 9.0 [d]	n.d. [d]	0.9–1.57	0.11–0.65
Cl^- $(10^{-2}$ M·kg$^{-1})$ [b]	16.4	n.d. [d]	9.0–15.0	3.4–4.1
Clay contents (%) [b]	55–58	59–65	15–36	16–31
Carbon (w%) [b]	1.1–1.2	0.85–1.1	1.9–2.8	2.1

Strain growth characteristics

Strain	F0	F3	F1	F2W	F2X	F2Y	G1
Temperature range (°C)	16–42	16–46	16–43	20–43	16–44	20–45	20–48
Optimum temperature	33–35	35–37	30–31	30–32	30–34	36–39	38–42
pH range	5.8–8.7	5.8–8.65	5.7–8.7	6.3–8.6	5.8–8.7	5.2–8.6	5.7–8.5
Optimum pH	6.8	6.85	6.9	7.0	6.95	6.75	6.9
NaCl range (g·l^{-1})	0–15	0–15	0–17	0–12	015	0–10	0–15
Optimum NaCl	5–7	5–10	10–15	3–7	7–14	1–7	1–7

[a] At 10–15 cm depth, at sampling.
[b] At 0–10 cm depth, on dry soil before sowing or transplanting of rice.
[c] At 10 cm depth, after 4 weeks of waterlogging.
[d] Moderately salted soil before first irrigation, then progessively desalting; n.d.: not determined.

ment-cultures on various electron donors (Fig. 1). Higher in vitro densities were obtained on lactate, after four or five successive transfers on the freshwater medium of Widdel and Pfennig [26], supplemented with 1 ml of trace-element solution SL7 [27] and reduced with 2 ml of a 0.5 M Na_2S solution (Medium 2). General techniques for the cultivation of strict anaerobes [28–31] were used for media preparations and during experiments. Substrates or vitamins were supplied from autoclaved or filter-sterilized stock solutions.

3.4. Isolation and purity control

Thirty fast-growing subcultures with a cell density higher than 10^9 were chosen for isolation tests (Fig. 1), using repeated deep agar (0.8–0.9%) dilutions adapted from Pfennig et al. [32]. Strain purity was checked in Medium 2 supplemented with 0.25% yeast extract (Difco), 0.25% peptone, 0.25% Biotrypcase (BioMérieux, Craponne, France), 0.25% nutrient broth, 0.25% glucose (or 0.25% fructose). Cultures were examined microscopically for purity after three weeks of incubation at 35°C.

3.5. Growth studies

Unless otherwise indicated, experiments were conducted in Medium 2 with 20 mM lactate and 20 mM sulfate, at 35°C and pH 7.2, and with 0.1% NaCl. Growth was monitored by measuring optical density at 580 nm with a Bausch and Lomb Spectronic 21 spectrophotometer. Total non-precipitated sulfides (H_2S, S^{2-}, and HS^-) were measured spectrophotometrically as colloical CuS [33].

The pH range of growth was estimated in bicarbonate buffered mineral Medium 2, where pH was adjusted with sterile Na_2CO_3, NaOH, or

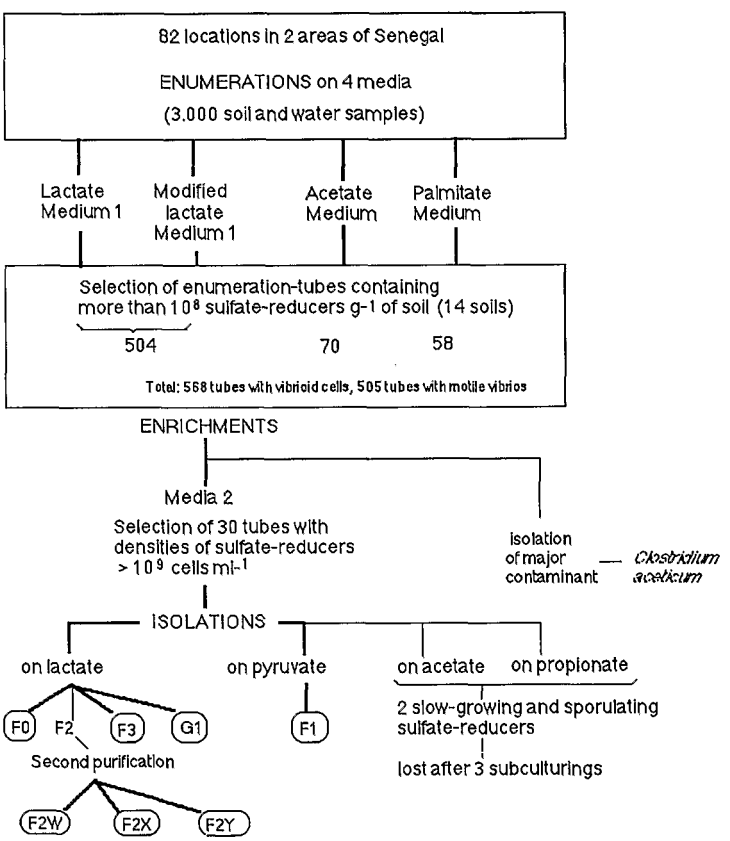

Fig. 1. Schematic representation of the isolation process of studied strains.

HCl solutions. The temperature range for growth was tested from 16 to 55°C. A possible salt requirement in addition to the concentration used in Medium 2 was tested by adding NaCl concentrations ranging from 0.1 to 3%.

Ability of strains to use various substrates as electron donor was tested in presence of 20 mM sulfate. When the growth failed, substrate oxidation ability was tested in presence of vitamins [32] and 2 g l^{-1} of yeast extract. Sulfite, thiosulfate, elemental sulfur, nitrate, fumarate, and malate were tested as electron acceptors in presence of 20 mM lactate.

3.6. Analytical techniques

Organic acids, alcohols, and fatty acids were assayed by high-performance liquid chromatography (HPLC): pump, Analprep 93 (Touzart et Matignon, Vitry-sur-Seine, France); flow rate, 0.6 ml/min; injection loop 20 μl; column ORH-801, 300 × 6.5 mm (Interaction Chemicals, Mountain View, CA); eluent: H_2SO_4 0.01N; H^+ ions exchange column, 8-μm pore size; elution according to the pK_a and/or the molecular mass for the non-ionic compounds; column temperature, 35°C; detection, differential refractometer (Knauer, Berlin, FRG); recorder, Chromatopak C-R3A (Shimadzu, Kyoto, Japan).

For cell pigment determination, 3 g of wet cells, suspended in 10 ml of 20 mM Tris · HCl buffer (pH 7.6), were sonicated and the suspension was centrifuged at 30 000 × g for 20 min. The resulting cell-free extract was centrifuged at 140 000 × g for 2 h. The pellet was resuspended in the previous buffer. Cytochromes were identified by recording air-oxidized and dithionite-reduced spectra, as well as the redox difference spectra of the dithionite-reduced minus air-oxidized of each fraction, using a Shimadzu UV 300 spectrophotometer (Shimadzu, Kyoto, Japan).

Gram staining was done using a standard method with a coloration kit (Sigma, St Louis, MO). *Escherichia coli* and *Micrococcus luteus* were used as controls.

The guanine-plus-cytosine content of the DNA (G + C%) was determined at the German Collection of Microorganisms (DSM, Braunschweig, FRG). DNA was isolated by chromatography on hydroxyapatite [34]. The G + C% was determined by HPLC; non-methylated Lambda virus DNA was used for calibration [35].

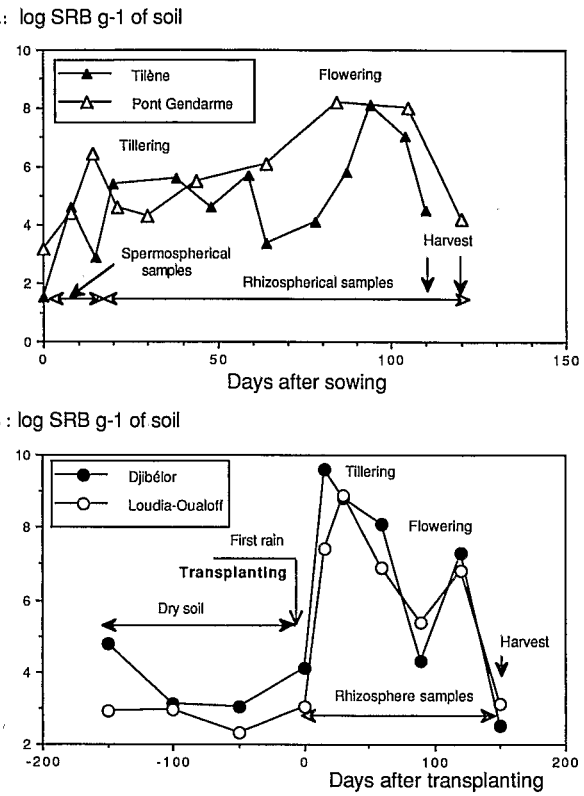

Fig. 2. (A) Lactate-using bacteria enumerated in the spermosphere and the rhizosphere of direct-seeded rice in 2 fields of the Senegal River delta (mean of 6 measurements). (B) Lactate-using bacteria enumerated before transplanting and in the rhizosphere of transplanted rice in 2 fields situated in Casamance/Senegal (mean of 6 measurements).

4. RESULTS

4.1. Field results

Densities of lactate-utilizing sulfate reducers were measured from sowing (spermospherical samples: 0–14 days) to harvest (rhizospherical samples: 15–110 or 120 days) in the two Senegal River delta soils (Fig. 2a). In the two Casamance soils, they were enumerated before the first useful rain (unplanted soil samples), and on rhizo-

spherical samples, from transplanting to harvest (Fig. 2b). Higher densities were observed on the days after seed germination, from transplanting to tillering, and at flowering.

4.2. Microscopical observations on enumeration and enrichment cultures

The enumeration-tubes chosen for enrichment-cultures (Fig. 1) contained two carbon

Table 2

Electron donors and acceptors, G+C mol %, pigments and morphology

Strain	F0	F1	F2W	F2X	F2Y	F3	G1	*D. vulgaris*	*D. desulfuricans*
Electron donors (in mM) on Medium 2									
Lactate (15) [a]	+	+	+	+	+	+	+	+	+
Pyruvate (15) [a]	+	+	+	+	+	+	+	+	+
Electron donors (in mM) oxidized on Medium 2, acetate (2 mM) and vitamins added [b]									
$H_2 + CO_2$ (2 bars)	+	+	+	+	+	+	+	+	+
Fumarate (10)	+	+	+	+	−	+	+	+	+
Malate (10)	−	+	+	+	−	+	+	−	+
Ethanol (5)	+	+	+	+	+	+	+	+	+
Propanol (5)	+	+	+	+	+	+	+	+	+
Butanol (5)	−	−	+	+	+	+	+	nr [c]	+
Glycerol (5)	(+) [d]	−	(+)	−	(+)	(+)	−	+	nr
Serine (10)	(+)	−	(+)	(+)	−	−	−	+	+
Choline (5)	−	−	−	−	−	−	−	−	+
Carbon substrates (in mM) fermented in sulfate-free Medium 2 [e]									
Pyruvate (15)	+	+	+	+	+	+	−	−	+
Malate (10)	−	−	−	−	−	−	−	+	−
Electron acceptors (in mM), lactate (20 mM) as electron donor									
Sulfate (20)	+	+	+	+	+	+	+	+	+
Sulfite (5)	+	+	+	+	+	+	+	+	+
Thiosulfate (10)	+	+	+	+	+	+	+	+	+
Elemental sulfur (10)	−	−	−	−	−	−	−	−	+
Nitrate (5)	−	−	−	−	−	−	−	−	+
Fumarate (10)	−	−	−	−	−	−	−	−	+
G+C mol % and pigments									
G+C mol %	62.5±0.6	59.0	60.4±0.2	62.8	56.3	60.1	60.2	61.0	55.0
Cytochromes	c_3	c_3	c_3	c_3	c_3	c_3	c_3	c_3	c_3
Desulfoviridin	+	+	+	+	+	+	+	+	+
Morphology									
Form	vibrio	vibrio	vibrio	vibrio	vibrio	vibrio	short rod [f]	vibrio	vibrio
Average length (μm)	3–6	4–7	3–7	2–6	3–6	4–7	2–4(4–6)	1.5–4	1.5–4
Average width (μm)	0.5–0.8	0.5–1.0	0.4–0.7	0.5–0.8	0.5–0.8	0.5–0.7	0.6–0.8 (0.8–1.0)	0.5–0.8	0.5–0.8

[a] Without vitamins or yeast extract.

[b] Substrates tested (mM), either in presence or absence of acetate or vitamins, and not supporting the growth of any of the 7 isolates: formate (10), oxalate (5), succinate (10), methanol (5), iso-propanol (5), iso-butanol (10), pentanol (10), acetate (10), propionate (10); n- or iso-butyrate (10), 2-methylbutyrate (10), n-benzoate (10), valerate (5) palmitate (2), fructose (15), glucose (10), xylose (5), ribose (5), and cysteine (10).

[c] nr = not reported.

[d] (+) Slow degradation without significant growth when yeast extract added (0.1%).

[e] No fermentative process observed, in sulfate medium without yeast extract on: lactate (15), succinate (10), fumarate (10), oxalate (10), fructose (15), glucose (10), and ethanol (10).

[f] Size and motility were different on lactate or on pyruvate (data in brackets).

sources, the electron donor (lactate, acetate, or palmitate) and yeast extract. Microscopical observations showed mixed cultures of one or more motile sulfate-reducing bacteria, and weakly motile or non-motile rods, sometimes observed in their sporulating stage. Vibrio-shaped cells (differing by motility and sizes) were significantly dominant in more than 90% of the tubes observed during the hours following the blackening of filter, whatever the site or the electron donor. Most of cultures (98%) did not show a modification of cell forms when subcultured on Media 1 or 2. But vibrios died when subcultured 1 to 3 times on acetate or palmitate-sulfate media. Four or five rapid subculturings on Medium 2 (free from yeast extract), with lactate or with pyruvate reduced the relative number of sporulating bacteria (that might be other sulfate reducers or contaminants, such as anaerobic bacteria using the yeast extract).

Enrichment-tests on propionate, acetate, *n*- and *iso*-butyrate, *n*-benzoate, palmitate, glucose and fructose failed, whereas those with propionate or palmitate progressively resulted in the selection of two sporulating sulfate-reducing bacteria (slighly curved rods or oval cells with round spores). They grew very slowly (2–6 months of incubation were necessary to obtain cell densities around 10^5), and could not be subcultured for more than three transfers and identified.

4.3. Isolation

Four strains (F0, F2, F3 and G1) were isolated on lactate (15 mM) as electron donor, while strain F1 was obtained on pyruvate (15 mM). They developed after 3–9 days of incubation at 35°C as brownish, lens-shaped colonies in deep agar tubes. Because the growth characteristics of strain F2 were not constant, this strain was repurified on deep-agar lactate medium, leading to the isolation of three strains (F2W, F2X, and F2Y).

4.4. Strain description

4.4.1 Morphology. The seven isolates were motile vibrios, 1–3 μm in length and 0.5–0.8 μm in width (Table 2). They lost motility and became spirilloid in old cultures. Strain G1 exhibited a slight variability (both in size and motility) when cultured on lactate or pyruvate. Cells stained Gram-negative and did not sporulate.

4.4.2. Physiology. Growth characteristics of the strains on Medium 2 are reported in Table 1. The range of temperature for growth was 16–48°C, optima were between 30 to 42°C. The range of pH was 5.2–8.7, optimum pH were between 6.75–7.0. Strains could grow in Medium 2 whithout additional NaCl. But NaCl improved growth, with optima observed when added concentrations varied from 1 to 5 g l^{-1}.

Data on electron acceptors are presented in Table 2. The strains were fast-growing bacteria with minimum doubling times between 1.5 and 4 h on Medium 2 with lactate or pyruvate. Lactate and pyruvate were incompletely oxidized to acetate, and growth occurred without addition of vitamins, yeast extract, peptone, or Biotrypcase. Fumarate (except for strain F2Y), malate, $H_2 + CO_2$, ethanol, propanol, and butanol (except for strains F0 and F1) could serve as electron donors only when vitamins and yeast extract were provided. Acetate was the end product of fumarate and malate degradation. Alcohols were degraded to the corresponding volatile fatty acids. Except strain G1, all isolates could ferment pyruvate. Substrates tested and not utilized are listed in Table 2.

Sulfate, sulfite, and thiosulfate were used as electron acceptors by all isolates (Table 2) and sulfide was the product of reduction. No isolate was able to use elemental sulfur, fumarate, malate, or nitrate as electron acceptor.

4.4.3. Cytochromes, pigments and DNA base composition. When reduced with sodium dithionite, the soluble extracts of all isolates exhibited absorption bands characteristic of c_3-type cytochrome, with maxima at 419, 522, and 552 nm. The oxidized extracts showed the cytochrome Soret peak at 408 nm. In addition, the spectra showed a characteristic absorption band at 628 nm indicating the presence of desulfoviridin [36,37].

The G + C% varied from 56.3 to 62.8 $\pm$ 0.3% (Table 2).

5. DISCUSSION

5.1. Taxonomy

The seven isolates are strictly anaerobic, mesophilic, Gram-negative, non-sporulating sulfate-reducing bacteria. They perform incomplete oxidation of lactate and pyruvate to acetate and CO_2. They are unable to oxidize acetate, propionate, butyrate, or palmitate. Cells contain c_3-type cytochromes and desulfoviridin. Based on the above characteristics, they belong to genus *Desulfovibrio* [1,3,36,38]. Morphological and physiological characteristics are similar to those of *D. desulfuricans* or *D. vulgaris* [1,3]. The G + C% of strain F2Y (56.3%) is close to that of *D. desulfuricans* (55%) whereas the G + C% of the other six strains (59–62.8%) is close to that of *D. vulgaris* (61%). The six strains similar to *D. vulgaris* differ from the type strain only by the use of glycerol (Table 2). Strain F2Y differs from *D. desulfuricans* by its inability to oxidize choline and to utilize elemental sulfur and nitrate as electron acceptors. Likely, strain F2Y is a strain of *D. desulfuricans* and other isolates are strains of *D. vulgaris*.

5.2. Low variability of isolated strains

Results show a limited strain diversity (two species of non-sporulating sulfate reducers) whereas we surveyed the rhizosphere of at least a dozen of rice cultivars growing in soils exhibiting a wide range of physico-chemical properties (Table 1) and situated at about 800 km distance. The dominance of non-sporulating sulfate reducers was also reported by Bak and Pfennig, in the sediment of Lake Constance, where fast-growing H_2- and lactate-consumer strains were mostly found [39].

However, reported field enumerations and subsequent isolations might be insufficient to conclude that rice spermosphere and rhizosphere are colonized only by the described fast-growing, mesophilic, neutrophilic, and non-sporulating strains.

Populations of acetate-utilizing sulfate reducers might be underestimated because of the short incubation time required to obtain positive results (blackening of the filter). Experiments by Jørgensen and Bak [40] on agar media with marine sediments showed that 12 weeks of incubation were necessary to obtain the maximum cell numbers of acetate-using sulfate reducers, whereas 1–3 weeks was enough for the development of all colonies of fast-growing lactate- (or H_2)-utilizing sulfate-reducing bacteria. When acetate- or palmitate-utilizing sporulating sulfate reducers were enumerated on modified Medium 1 (lactate replaced by 15 mM acetate, or 1 mM palmitate), 2–4 weeks of incubation were needed to obtain the blackening of the filters.

The low strain diversity recorded could also be attributed to low salt content of the media used for enumeration, enrichment and isolation. In particular, fatty acid-oxidizing sulfate-reducing bacteria (*Desulfobacter* or *Desulfobacterium* spp.) require a relatively high level of NaCl. Laanbroek and Pfennig [41], comparing the anaerobic oxidation of short-chain fatty acids in freshwater and in marine sediments, established that acetate-oxidizing strains did not grow in freshwater medium. They were isolated from brackish or marine samples only, and their densities were significantly lower than those of *Desulfovibrio desulfuricans*.

Vitamins requirement could have limited the growth of some strains. However, during the last survey in Loudia Oualoff, the use of the modified Medium 1 (yeast extract replaced by biotin and vitamin B_{12}), did not result in the growth of morphologically different sulfate reducers, as compared with samples collected during the preceding cropping season. But it reduced the relative number of non-sulfate-reducing bacteria—probably those able to use yeast extract as carbon source—and increased the number of vibrioid sulfate reducers.

Acetate- or palmitate-utilizing sporulating sulfate reducers, found as minor component in less than 10% of fast-growing enumeration tubes, were different from *Desulfobacter postgatei* or *Desulfotomaculum acetoxidans*, as shown by the very slow growth observed on the specific acetate-sulfate media established for these two species by Pfennig et al. [26,42]. Enrichment-tests with various times of incubation, carbon sources, salt content,

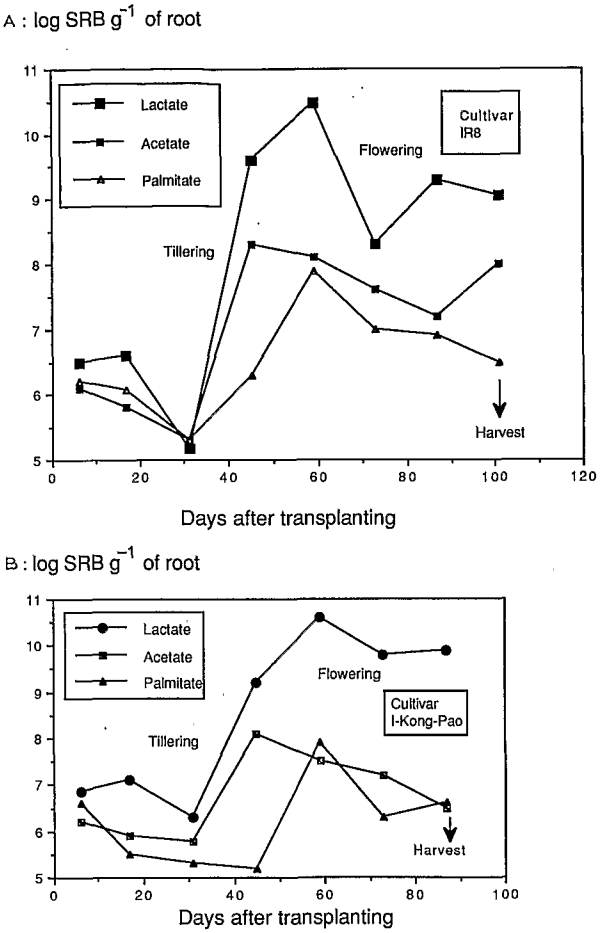

Fig. 3. (A) Lactate-, acetate-, and palmitate-using bacteria enumerated on the rhizoplane of transplanted IR8 rice in Loudia-Oualoff fields, Casamance/Senegal (mean of 6 measurements). (B) Lactate-, acetate-, and palmitate-using bacteria enumerated on the rhizoplane of I-Kong-Pao transplanted rice in Loudia-Oualoff fields, Casamance/Senegal (mean of 6 measurements).

and pH, did not permit to isolate these species [21].

In a field experiment [11,18], it was shown that densities of acetate- or palmitate-utilizing sulfate reducers, were 10^2–10^3 times less abundant than fast-growing lactate-utilizing sulfate reducers on the root surface (Figs. 3a,b). Because of their low populations and very low growth rate, it was assumed that they were not significantly implicated in rice plant intoxication. Other sulfate reducers resembling *Desulfobulbus* (oval or lemon shape), or *Desulfonema* (filamentous cells) were also observed as rare contaminants [11]. Their isolation was not assayed: they could not be responsible for the damages on rice because of their very low occurrence in the rhizosphere.

Despite possible methodological reservations, the dominance of non-sporulating sulfate reducers observed in this study is consistent with a previous work reporting that the detrimental effects of sulfides produced in ricefieds was due, whatever the soil and the cultivar, to lactate-utilizing sulfate reducers, and may be indirectly correlated with their number. Jacq and Roger [20] determined that sulfide toxicity was the result of the antagonistic action of sulfate-reducing, lactate-oxidizing bacteria versus sulfide-oxidizing bacteria. Major injuries to and death of the rice plant occurred only when the density of lactate-oxidizing sulfate reducers, enumerated on Medium 1, was at least 100 times higher than that of *Thiobacillus denitrificans* in the same sample.

It was found that non-sporulating sulfate reducers were associated in the rhizosphere of diseased rice plant with O_2-consuming facultative ferric iron-reducing bacteria, including Clostridia [11,21]. We also found that major contaminants on lactate-sulfate were mainly Clostridia. *Clostridium aceticum* was evidenced as the dominant species [21]. As it did not use lactate, it was supposed that its presence did not significantly modify the composition of lactate-Medium 1 during a short incubation time.

5.3. Strains and their environment

Isolated strains grow in rice rhizosphere on the small number of carbon substrates (Table 2) that can be provided by germinating seed and root exudates [9,13,43], or/and resulting from the activity of heterotrophs. Lactate and pyruvate exudated by rice roots or germinating seeds are the main electron donors for all strains and can ensure their growth. Experiments with hydroponic cultures of 1–2-week-old seedlings of IR8 rice [43] showed that lactate + pyruvate comprised about 15% of excreted carbon compounds and only 25–30% of substrates were usable by most *Desulfovibrio* strains. Average exudation was esti-

mated to be 3.2 μg of lactate and 2.1 μg of pyruvate day^{-1} seedling^{-1}. Fumarate and malate, oxidized by most of the studied strains only in presence of vitamins, represented 8.3 and 1.2 μg day^{-1} seedling^{-1}, respectively.

The surveys preceding this study did not establish any minimum sulfate content in soils that could be required for the development of sulfide toxicity [21]. Such result may be partly explained by high densities of *Thiobacillus denitrificans* in rice rhizosphere [4,20,26], which may contribute to a rapid turnover of reduced sulfur compounds, providing enough sulfates for *Desulfovibrio* activities [21]. Brandl et al. [44] reported that *Desulfovibrio* spp. were very active in the sulfate-limited sediment of Lake Geneva.

Concentrations of NaCl used for growing isolates from brackish ricefields (5–15 g l^{-1}) were similar to or higher than the concentrations in soil solution allowing rice growth (1–2 g l^{-1} at sowing or transplanting stages to 5–6 g l^{-1} at flowering). Plot experiments with saline soil of Tilène 3 showed that salinity in the spermosphere and the rhizosphere decreased from 0.2 g l^{-1} at sowing stage to 0.09 g l^{-1} at flowering stage [16]. Field surveys [21] also showed that NaCl measured in situ under the plow layer with a permanent piezometer, did not exceed 3–4 g l^{-1}. Therefore, it can be assumed that sulfate reducers in the rhizosphere and spermosphere grow at low salinity level (0.2–3-4 g l^{-1}). Whereas laboratory results showed that no isolates required additional NaCl for growth, the optimum NaCl range was significantly higher than the concentration generally used for growing freshwater strains. This agrees with the high densities of *Desulfovibrio desulfuricans* found in brackish sediment [41] and with the hypothesis of a brackish origin of *Desulfovibrio* occurring in ricefields established on former mangrove soils [21,22]. However, it does not explain why sporulating and completely oxidizing sulfate reducers usually occurring in the brackish environments were not encountered in ricefields established on mangroves.

Isolates were neutrophilic, while they were isolated from soils having an initial pH ranging from 3.6 to 6.25 after rewetting [13,21]. However, flooding results in an increase of soil pH by 1.5 to 2.0 units during the 3–4 weeks following submersion [11,13,16,23]. A survey in Senegal River delta showed that 95% of rhizospheric pHs were between 6.5 and 7.2, and that the pH in blackened rhizosphere was 0.4 to 0.6 units higher than in sulfide-free soil [21]. Sulfate reducers grow under neutral conditions when they are close to the roots: optimum pH ranges established for the seven isolates cover pH range measured in situ in rice rhizosphere.

All strains were mesophilic. During the rice cultivation cycle in Senegal (July to November), air temperature varied from 23–24°C (night) to 37–38°C (day), maximum water temperature was generally 30–32°C under the rice canopy, temperature at the soil surface sometimes reached 45°C, and soil temperature at 15 cm depth remained around 28–30°C [21,23]. The temperature range of isolates covered temperatures that were measured in soil and floodwater. In situ measurements indicated that sulfate reducers activity in the rice spermosphere and rhizosphere developed around 30 $\pm$ 2°C. This range is lower than that recorded in vitro (Table 1). Upper temperature allowing growth (42–48°C) explains why sulfate-reducing activity can be detrimental to germinating seeds at the soil surface (2–3 cm depth), where temperature is not buffered, and why sulfate reducers can also survive marked increases in temperature in the floodwater.

6. CONCLUSIONS

Field surveys showed the presence of high densities of fast-growing, lactate-using, sulfate reducers in the rhizosphere of rice plant presenting sulfide-toxicity symptoms. The seven sulfate-reducing bacteria isolated from rice rhizosphere belonged to the *Desulfovibrio* group. They are characterized by a low number of electron donors, which are provided by rice exudates and incompletely oxidized. They are fast-growing strains, which may explain why they could damage in a few days germinating seeds, seedlings and even maturing plants at reproductive stage.

Their densities in sulfide-intoxicated rice rhizosphere and spermosphere, and in enrichment

cultures, were significantly higher than those of sporulating sulfate reducers which could not be identified. Therefore, described *Desulfovibrio* are to be considered as mostly responsible for the development of sulfide toxicity.

In the studied area, environmental conditions in rice rhizosphere appear to be quite similar in terms of available substrates, temperature, salinity and pH. *Desulfovibrio* seems to be the genus best adapted to conditions prevailing in this environment.

The seven strains have been deposited with the D.S.M. under DSM numbers: 6617 (F0), 6618 (F1), 6619 (F2W), 6620 (F2X), 6621 (F2Y), 6622 (F3) and 6623 (G1).

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