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Identification of thiosulfate- and sulfur-reducing bacteria unable to reduce sulfate in ricefield soils

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Abstract − Using peptides as energy sources, H_2 as electron donor, thiosulfate as electron acceptors, we isolated, from four ricefield soils originating from France and the Philippines, 52 strains of anaerobes, among which 18 reduced thiosulfate but not sulfate. These 18 strains were strict proteolytic asaccharolytic anaerobes producing H_2S when grown on thiosulfate + H_2. They exhibited the same restriction fragment length polymorphism (RFLP) profile (11 restriction enzymes tested). Partial sequencing of the 16S rDNA showed that they belonged to the genus *Clostridium* and were phylogenetically related to *C. subterminale*. DNA–DNA hybridization of a representative strain with the closest *C. subterminale* strain (DSM 6970^T) yielded a value of 68.9%. Previous counts of thiosulfate reducers unable to reduce sulfate (TSRnSR) in ricefield soils, their identification as *Clostridium* strains, and the known ubiquity of this genus in such soils indicate that TSRnSR of the genus *Clostridium* may play a significant role in S cycling in some wetland soils. © 2001 Éditions scientifiques et médicales Elsevier SAS

Clostridium **/ microflora / rice / soil / sulfate / thiosulfate**

1. INTRODUCTION

The sulfur cycle in rice soils has two major aspects: plant nutrition and sulfide toxicity to the plant. Sulfide toxicity in ricefields was exclusively attributed to the activity of sulfate-reducing bacteria [13], which can reduce both sulfate and thiosulfate, until we provided the first evidence that thiosulfate reduction can be performed in rice soils under anaerobic conditions by non-sulfate-reducing bacteria [10]. We counted in five ricefield soils, by the MPN method, culturable thiosulfate-reducing bacteria unable to reduce sulfate (TSRnSR), using peptides as energy sources, H_2 as electron donor and thiosulfate as electron acceptor. Their densities in dry soils ranged from 4×10^1 to 4×10^4 cells g^1 and were of the same order of magnitude as sulfate-reducers using lactate, suggesting that TSRnSR may play a significant role in S cycling in some wetland soils. The goal of this study was to characterize the bacteria that were previously evidenced [10]. In a first step 52 anaerobic strains were isolated from four ricefield soils using various enrichment procedures and selective media. Among them 18 were thiosulfate reducers unable to reduce sulphate (TSRnSR) that were broadly characterized. Then two strains (25 and 45), selected as representative of the TSRnSR group were used for a more accurate physiological and taxonomical characterization.

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2. MATERIALS AND METHODS

2.1. Ricefield soils

The soils used originated from ricefields from South of France (Camargue) and the Philippines (Maahas, Maahas alkaline, and Tiaong). Their properties and the method of sampling were described by Joulian et al. [15]. Camargue and Maahas soils were selected according to a previous study [10] which showed that their TSRnSR populations ranged from 10^3 to 10^4 g^1 dry soil. Camargue soil is a moderately alkaline silt

fine clay (pH 7.7); Maahas is a sligtly acidic clay (pH 5.9); Maahas alkaline is a Maahas soil artificially alkalinized in situ for several years by carbonate addition (pH 7.9). Tiaong, an alkaline soil (pH 7.8) rich in organic matter, was used because the previous study [10] indicated a positive correlation between soil pH and the abundance of TSRnSR and hydrogenotrophic sulfate-reducers.

2.2. Culture media

The basal medium, derived from Ravot et al. [22], contained per liter of distilled water: 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, 0.1 g; resazurin 0.1%, 1 mL ; Balch trace-element solution, 10 mL [4]. The pH was adjusted to 7.0 with a 10 N KOH solution. Media were distributed in Hungate's tubes (4.5 mL per tube) previously flushed and filled with N_2/CO_2 (80/20; v /v) [14]. After autoclaving (45 min at 110°C), sterile stock solutions of 2% $Na_2S.9H_2O$ and 10% $NaHCO_3$ were added in each tube to a final concentration of 0.08 % (w/v) and 0.5 % (w/v), respectively. Na_2S was used as reducing agent and resazurin as redox potential indicator. Final pH of the medium was 7.0. Eight media, prepared by subsequent addition to basal medium of the following substrates and electron sources and acceptors, were used to isolate and characterize the strains:

– M1: 5 g.L^{-1} Biotrypcase + 20 mM thiosulfate (to evidence non-H_2-utilizing thiosulfate-reducers that could be inhibited by H_2)
– M2: 5 g.L^{-1} Biotrypcase + 20 mM thiosulfate + 2 bars H_2 (to evidence H_2-utilizing thiosulfate-reducers)
– M3: 5 g.L^{-1} Biotrypcase + 20 g.L^{-1} S° (to evidence sulfur-reducers)
– M4: 20 mM lactate + 20 mM SO_4^{-2} (to evidence sulfate-reducers using lactate)
– M5: 5 g.L^{-1} Biotrypcase + 20 mM thiosulfate under an air atmosphere (to test for strict anaerobes)
– M6: 20 mM glucose (to test for fermentative activity)
– M7: 5 g.L^{-1} Biotrypcase (as control)
– M8: 5 g.L^{-1} Biotrypcase + 20 mM sulfate

2.3. Inocula used for isolations

Four kind of inocula were used to isolate TSRnSR:

Soils suspensions (10 g soil d.w. + 20 mL M1) incubated under 2 bars H_2 for 15 days at 37°C, which favored TSRnSR and H_2-utilizing sulfate-reducing bacteria unable to oxidize lactate [10].

Soils suspensions as above, but incubated only under N_2/CO_2 (80/20, v/v) without H_2 to allow the growth of the TSRnSR that could be inhibited by H_2.

Soils suspensions as above, but not preincubated, to avoid strain selection by the medium during preincubation.

The last positive MPN tubes previously obtained from TSRnSR counts in Maahas, Maahas alkaline, and Camargue soils [10]. These tubes contained initially 2 bars H_2.

2.4. Preselection and purification of the strains

The above inocula were used to prepare serial suspension-dilutions (1/10) in roll-tubes [14], with M1 medium solidified with 1.5 % agar in order to obtain colonies. According to the pretreatments used to prepare the inocula, tubes with inocula 1, 3, and 4 were filled with 2 bars H_2 while tubes with inoculum 2 were filled only with N_2/CO_2 (80/20, v/v). Tubes were incubated at 37°C for one week. All individual colonies that developed in the two last positive tubes were collected under anaerobic conditions and homogenized separately in basal medium. Each suspension was used to inoculate four tubes containing media M1 to M4. Tubes were incubated for three weeks at 37°C and tested for growth and H_2S production. Further isolation was performed by the roll-tube technique [14] on the tubes showing a statistically significant H_2S production on M1 or M2 (*figure 1*).

2.5. Metabolic characterization of the strains

The isolated pure strains were grown in 20 mL flasks on media M1 to M7. Tubes were incubated for two weeks at 37 °C and tested for growth and H_2S production. The effect of electron acceptors on growth was studied in triplicate tubes on media M1 (thiosulfate) and M8 (sulfate) as compared with M7 (control).

Representative strains were further studied for additional metabolic features. Substrates for growth were tested in basal medium with 1 g.L^{-1} of yeast extract (Difco Laboratories, Detroit, MI), 20 mM sodium thiosulfate. Amino acids were tested at 10 mM, peptides and proteins (gelatin, casamino acids) at 2 g.L^{-1}, and sugars (cellobiose, mannose, xylose, fructose, ribose, glucose, lactose) at 20 mM. Temperature, pH, and sodium chloride ranges and optima were determined in duplicate Hungate tubes containing basal medium with 1 g.L^{-1} Biotrypcase and 5 g.L^{-1} gelatin. pH range was obtained by injecting $NaHCO_3$ or Na_2CO_3 from 10% sterile anaerobic stock solutions. Sodium chloride was weighed directly into Hungate tubes before adding the medium. Strains were subcultured at least twice under the same conditions. Growth was checked by measuring OD at 580nm.

2.6. Analytical techniques

Bacterial growth was measured at 580 nm with an UV 160A Shimadzu spectrophotometer. Sulfide production was determined colorimetrically as described by Cord-Ruwish [8]. Fatty acids were detected by high-performance liquid chromatography, using an ORH 801 column (250 mm in length, 4.1 mm in

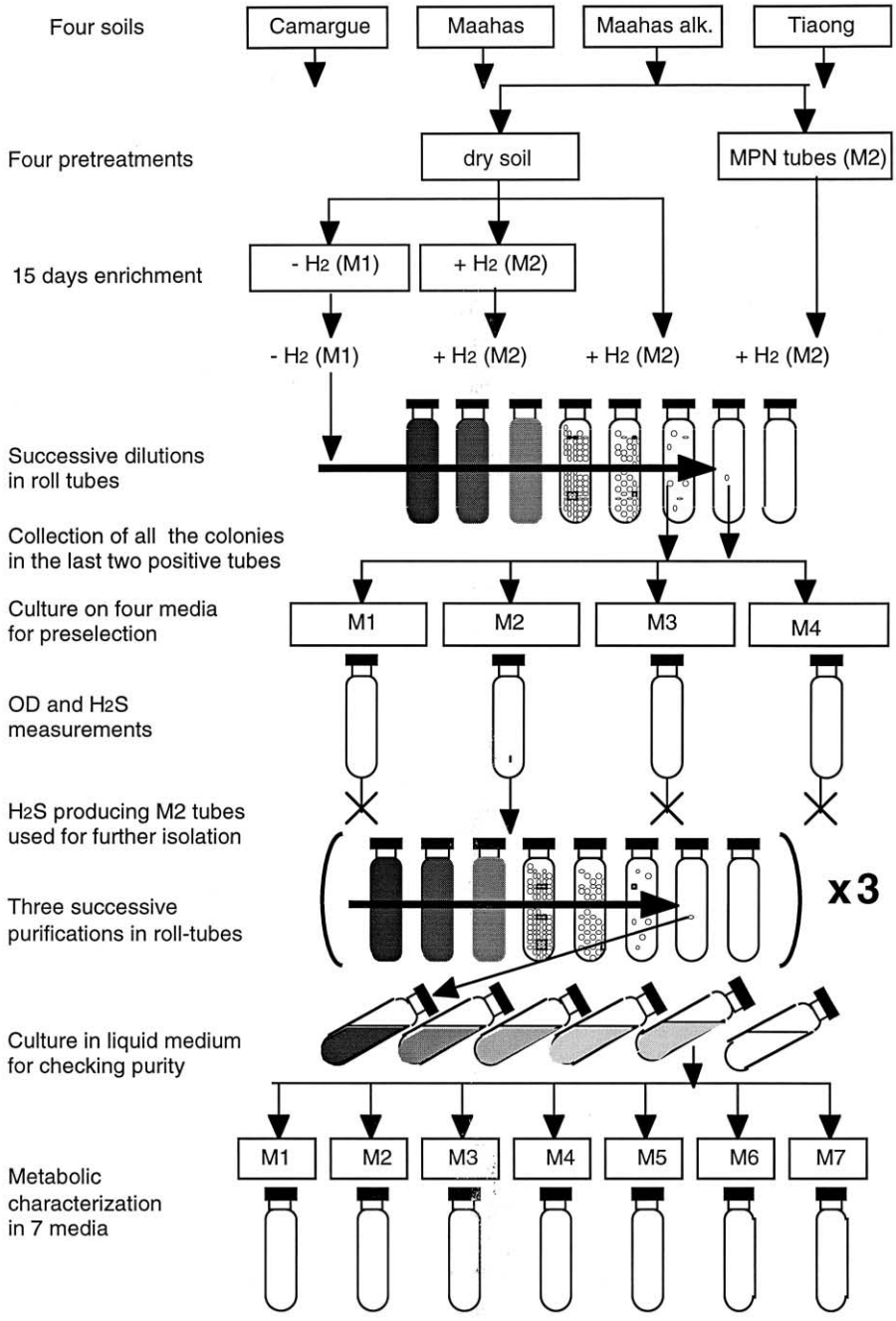


Figure 1. Purification/characterization process of the strains. Media composition: M1: Biotrypcase (BT) + thiosulfate (Thio); M2: BT+Thio+H_2: M3: BT+S°; M4: Lactate+SO_4; M5: BT+Thio+O_2; M6: Glucose; M7: BT.For concentrations see section 2.2 in the text.

diameter; Interaction Chemicals Inc., Mountain View, CA) and a RID-6A Shimadzu detector. Hydrogen production was determined with a GC-8A Shimadzu gas chromatograph. Electron microscopy observations were performed as described by Fardeau et al. [11].

2.7. DNA extraction and amplification of 16S rDNA

DNA was extracted from the isolates as described by Andrews and Patel [2]. The universal primers Fd1

and Rd1 [9] were used to obtain a PCR product of approximately 1.5 kb corresponding to base positions 8 to 1542, based on *E. coli* numbering of the 16S rDNA [27]. PCR products were purified with a Wizard DNA Clean-Up System column (Promega).

2.8. Digestion of 16S rDNA by restriction enzymes

The 16S rDNA of the 52 anaerobic strains isolated was digested by 11 restriction enzymes possessing four (*Cfo* I, *Alu* I, *Tru 9*I, *Taq* I, *Rsa* I, *Sau* 3AI and *Hae* III), five (*Sau*96I), and six (*Cla* I, *Pvu* I and *Xba* I) nucleotides restriction sites. Restriction was performed in 10 µL reaction medium containing 1 µL of 10X enzyme buffer, 0.2 U of endonuclease (Promega), 3.8 µL water, and 5 µL of PCR product (100 ng/µL). It was incubated for one hour at adequate temperature, in 200 µL Eppendorf tubes. Restriction fragment length polymorphism (RFLP) patterns were analyzed by horizontal gel electrophoresis at 80 V for h.

2.9. Direct sequencing of PCR products

PCR product were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing kit with Ampli Taq FS DNA polymerase and an ABI 373A sequencer, as described by Baena et al. [3]. F3 and R6 primers were used for partial sequencing. Fd1, F2, F3, F4, Rd1, R2, R5 and R6 primers were used for total sequencing [9]. Sequences were assembled, aligned to almost half-length consensus 16S rDNA gene sequence and checked for accuracy manually, using the alignment editor ae2 [18]. They were compared to other sequences in GenBank Database [5] using BLAST [1], and in the Ribosomal Database Project version 5.0 using SUGGEST_TREE [18]. Positions of sequences and alignment uncertainties were omitted from the analysis. Pairwise evolutionary distances based on unambiguous nucleotides were computed using DNA-DIST (Jukes & Cantor option) and neighbour-joining programs of the PHYLIP suite of programs [12]. Five hundred and fifty two unambiguous nucleotides were used for partial sequencing and 1365 for total sequencing of 16S rDNA.

2.10. Determination of G + C content and DNA-DNA hybridization

Both analysis were performed by the DSMZ (Deutsche Sammlung von Mikroorganismen and Zelkulturen, Braunschweig, Germany) as previously described [19].

2.11. Nucleotide sequence accession numbers in the GenBank database

Reference strains: *Clostridium argentinense* (ATCC 27322), X68316 ; *C. bifermentens* (DSM 10716), X73437 ; *C. botulinum* A (NCTC 7272), X68185 ; *C. botulinum* B (NCTC 7273), X68186 ; *C. botulinum* F (ATCC 25764), L37593 ; *C. botulinum* G (strain 113/30), X68317 ; *C. difficile* (DSM 11209), X73450 ; *C. felsineum* (DSM 794), X77851 ; *C. fervidum* (ATCC 43204), L09187 ; *C. glycolicum* (DSM 1288^T), X76750; *C. grantii* (DSM 8605), X75272 ; *C. halophilum* (DSM 5387), X77837 ; *C. histolyticum* (ATCC 19401), M59094 ; *C. intestinale* (DSM 6191^T), X76740 ; *C. irregularis* (DSM 2635), X73447 ; *C. lituseburense* (ATCC 25759), M59107 ; *C. paradoxum* (DSM 7308^T), Z69941 ; *C. pascui* (DSM 10365), X96736 ; *C. sordelli* (ATCC 9714), M59105 ; *C. sporogenes* (ATCC 3584), X68189 ; *C. subterminale* 2 (ATCC 25774), X68451 ; *C. subterminale* 4 (ATCC 25774), L37595 ; *C. tetani* (NCTC 279), X74770 ; *C. tetanomorphum* (NCIMB 11547), S46737 ; *C. thermoalcaliphilum* (DSM 7309), L11304 ; *Escherichia coli*, A14565 ; *Eubacterium tenue* (ATCC 25553), M59118.

16S rDNA sequences determined in this study: (1) isolated strains (4, 8, 15, 17, 19, 21, 22, 23, 26, 27, 30, 31, 32, 36, 38, 39, 44, 46, 47, 48, 49, 50, 52, 6, 25, and 45) under the accession numbers AF191226 to AF191251 respectively ; (2) collection strains: *Clostridium subterminale* (DSM 758), AF241843 ; *C. subterminale* (DSM 2636), AF241842 ; *C. subterminale* (DSM 6970^T), AF241844.

3. RESULTS

3.1. Preselection and purification of the strains

Thresholds of H_2S determined in the liquid phase of uninoculated controls were 1.5 mM for media M1, M2, and M4 and 2 mM for medium M3 (containing S°).

The first step of the isolation procedure allowed to pick up 136 colonies, not necessarily axenic. Their screening through (i) H_2S production and growth after three weeks of incubation at 37°C on M1 and M2 (indicating the growth of thiosulfate reducers, with or without H_2), and (ii) the record of absence of H_2S production on M4 (indicating the inability to reduce sulfate with lactate), allowed to select tubes containing TSRnSR. Among the 136 colonies:

– 106 (78%) of those tested on S° (M3) produced more than 2 mM H_2S, indicating that they contained sulfur-reducers, some of which can also reduce thiosulfate.

– growth on M4 occurred in 20 tubes (22%), among which only four reduced sulfate to H_2S, which confirmed the selectivity for nonsulfate-reducing bacteria of a medium containing thiosulfate and Biotrypcase, both in the presence and the absence of H_2.

– only 52 produced significant quantities of H_2S in the presence of H_2, thus suggesting the presence of H_2-utilizing thiosulfate reducers in these tubes. After further purification, 52 axenic strains were obtained, one from each of these 52 tubes, and were characterized for specific metabolic properties.

3.2. Metabolic characterization of the strains

Growing the 52 isolates on M1 to M7 yielded the following results (*table I*):
– all strains grew on thiosulfate + Biotrypcase (M1) without producing H_2S,
– all strains grew on S° + Biotrypcase (M3), producing significant quantities of H_2S (> 2 mM),
– 18 strains grew on thiosulfate + Biotrypcase + H_2 (M2), producing significant quantities of H_2S (> 1.5mM), indicating their probable ability to oxidize H_2,
– all strains grew on Biotrypcase (M7), but on the average, the OD measured after 14 days of incubation was significantly lower for the 18 strains that produced H_2S on M2,
– no strain grew under aerobic conditions (M5),
– 23 strains did not utilize glucose (M6), and
– two strains possibly grew on lactate + sulfate (M4) but did not produce H_2S.

This indicates that the 52 isolates were sulfur-reducers, among which 18 were TSRnSR. The dominance of sulfur-reducers among the isolates most probably resulted from their ability to grow on Biotrypcase in the absence of S°. S° might also have been an intermediary product of thiosulfate-reduction during bacterial enrichments, since some anaerobes can reduce thiosulfate into S° [17]. The 18 TSRnSR strains originated from the four soils; eight were obtained from inocula preincubated with H_2, four from inocula preincubated without H_2, two from dry soils, and four from the MPN tubes of Maahas and Camargue soils.

The study of the effect of electron acceptors through growth on M1, M7, and M8 showed that:
– the maximum OD_{580nm} of the cultures of the 18 TSRnSR strains remained lower than 0.38 on Biotrypcase (M7) and Biotrypcase + SO_4^{2-}(M8). The average ratio of OD_{580nm} with and without sulfate was 1 (standard error (s.e.) = 0.2), showing that sulfate was not used as electron acceptor,
– thiosulfate addition to the medium, increased the growth of the 18 TSRnSR strains by more than 20%, but not that of the 34 other sulfur-reducing strains.

3.3. Digestion of 16S rDNA by restriction enzymes

Digestion bands were obtained with all but one restriction enzymes (*Cla*I), which allowed to classify strains within nine RFLP groups (*table I*). All the TSRnSR strains belonged to group I.

3.4. Direct sequencing of PCR products

Twenty six strains of sulfur- and thiosulfate-reducers were chosen for sequencing among the 52 isolated strains. They were selected to be representative of the nine RFLP groups and the four soils (*table I*). For each strain, about 600 bp of 16S rDNA were sequenced. BLAST programme was used to select, from GenBank and EMBL databases, sequences close to those obtained. Analysis showed that the 26 strains belonged to the order *Clostridiales* and to genera *Clostridium* and *Eubacterium* (*figure 2*). The TSRnSR strains were close to *C. subterminale* with 91 to 98 % similarity.

3.5. Additional characterization of two TSRnSR strains

Strain 25 originated from Maahas alkaline soil, and strain 45 from Tiaong soil. Both strains were motile rods, 5 by 1 µm, with terminal or subterminal spores and a Gram-positive type of cell wall ultrastructure (*figures 3a and b*).

3.5.1. Metabolic properties

Both strains did not utilize sugars. They grew best on gelatin, exhibiting the same final OD_{580nm} after 48h. Strain 25 grew four-time faster on gelatin than on casamino acids, which were not used by strain 45. Both strains grew on pyruvate but not on lactate. The two strains exhibited a similar metabolism of amino-acids (AA) (*table II*). Both did not utilize acid AA (aspartate and glutamate) while they used basic AA, especially lysine. On lysine, histidine, and serine they grew without the addition of thiosulfate. Growth on others AA required the addition of thiosulfate. HPLC analysis of AA degradation products showed that acetate was produced from alanine, serine, threonine, lysine, arginine, cysteine, and asparagine. Other degradation products recorded were isobutyrate from valine, methyl-2-butyrate from isoleucine, isovalerate from leucine, butyrate from lysine, propionate and unidentified compounds from methionine and histidine together with acetate. No sulfide was produced except for strain 45 growing on asparagine, cysteine, and alanine, with which levels remained low (2.0 – 2.6 mM). Strains 25 and 45 produced H_2 (1 mM) only on arginine.

3.5.2. Optimum pH and temperature

Optimum pH and temperature were determined on basal medium containing 1 $g.L^{-1}$ Biotrypcase and 5 $g.L^{-1}$ gelatin, because it allowed the best growth during metabolic tests. As previous experiments showed that both strains grew in the absence of H_2, no H_2 was added in the tubes, to avoid a possible inhibition.The optimum temperature for growth was 34°C for both strains. Growth occurred between 10 and 50°C for strain 25 and between 15 and 45°C for strain 45. The optimum pH was 8.2 and growth occurred between 5.5 and 9.5 for both strains. They grew with no NaCl added and tolerated up to 50 $g.L^{-1}$.

3.5.3. Effect of thiosulfate and H_2 on growth

Thiosulfate addition allowed strains to reach a higher OD_{580nm} at the stationary phase (+27 % for

Table I. Origin, treatment, growth, H_2S production, and RFLP group of the S2 isolated strains.

No.	Origin	Treatment	Growth OD (580nm)							H_2S production				Cluster
			M1	M2	M3	M4	M5	M6	M7	M1	M2	M3	M7	
2	C	+ H_2	+	+	+	–	–	–	+	–	+	+	–	I
5	C	+ H_2	+	+	+	–	–	–	+	–	+	+	–	I
6	C	MPN	+	+	+	–	–	–	+	–	+	+	–	I
7	C	+ H_2	+	+	+	–	–	–	+	–	+	+	–	I
8	C	+ H_2	+	+	+	–	–	–	+	–	+	+	–	I
9	M	MPN	+	+	+	–	–	–	+	–	+	+	–	I
10	M	MPN	+	+	+	–	–	–	+	–	+	+	–	I
11	M	+ H_2	+	+	+	–	–	–	+	–	+	+	–	I
12	M	+ H_2	+	+	+	–	–	–	+	–	+	+	–	I
13	M	+ H_2	+	+	+	–	–	–	+	–	+	+	–	I
14	M	MPN	+	+	+	–	–	–	+	–	+	+	–	I
15	M	– H_2	+	+	+	–	–	–	+	–	+	+	–	I
16	M	– H_2	+	+	+	–	–	–	+	–	+	+	–	I
18	M	– H_2	+	+	+	–	–	–	+	–	+	+	–	I
25	MA	– H_2	+	+	+	–	–	–	+	–	+	+	–	I
41	T	dry	+	+	+	–	–	–	+	–	+	+	–	I
44	T	dry	+	+	+	–	–	–	+	–	+	+	–	I
45	T	+ H_2	+	+	+	–	–	–	+	–	+	+	–	I
24	MA	+ H_2	+	+	+	–	–	–	+	–	–	+	–	II
26	MA	MPN	+	+	+	+	–	–	+	–	–	+	–	II
49	T	dry	+	+	+	–	–	–	+	–	–	+	–	II
38	MA	dry	+	+	+	–	–	–	+	–	–	+	–	II
21	M	dry	+	+	+	–	–	+	+	–	–	+	–	III
28	MA	dry	+	+	+	–	–	+	+	–	–	+	–	III
29	MA	dry	+	+	+	–	–	+	+	–	–	+	–	III
31	MA	dry	+	+	+	–	–	+	+	–	–	+	–	III
39	MA	– H_2	+	+	+	–	–	–	+	–	–	+	–	IV
1	C	+ H_2	+	+	+	–	–	+	+	–	–	+	–	V
3	C	+ H_2	+	+	+	–	–	+	+	–	–	+	–	V
33	MA	dry	+	+	+	–	–	+	+	–	–	+	–	V
34	MA	dry	+	+	+	–	–	+	+	–	–	+	–	V
35	MA	dry	+	+	+	–	–	+	+	–	–	+	–	V
36	MA	dry	+	+	+	–	–	+	+	–	–	+	–	V
37	MA	dry	+	+	+	–	–	+	+	–	–	+	–	V
43	T	dry	+	+	+	–	–	+	+	–	–	+	–	V
40	T	dry	+	+	+	–	–	+	+	–	–	+	–	V
42	T	dry	+	+	+	–	–	+	+	–	–	+	–	V
50	T	dry	+	+	+	–	–	+	+	–	–	+	–	V
4	C	+ H_2	+	+	+	–	–	+	+	–	–	+	–	V
51	T	dry	+	+	+	–	–	+	+	–	–	+	–	V
52	T	dry	+	+	+	–	–	+	+	–	–	+	–	V
27	MA	MPN	+	+	+	–	–	+	+	–	–	+	–	VI
30	MA	dry	+	+	+	–	–	+	+	–	–	+	–	VI
32	MA	dry	+	+	+	–	–	+	+	–	–	+	–	VII
46	T	+ H_2	+	+	+	+	–	+	+	–	–	+	–	VII
17	M	– H_2	+	+	+	–	–	+	+	–	–	+	–	VII
23	MA	+ H2	+	+	+	–	–	+	+	–	–	+	–	VII
47	T	– H_2	+	+	+	–	–	+	+	–	–	+	–	VII
48	T	– H_2	+	+	+	–	–	+	+	–	–	+	–	VII
19	M	+ H_2	+	+	+	–	–	+	+	–	–	+	–	VIII
20	M	dry	+	+	+	–	–	+	+	–	–	+	–	VIII
22	M	dry	+	+	+	–	–	+	+	–	–	+	–	IX

C: Camargue; M: Maahas; MA: Maahas alkaline; T: Tiaong; (M1: Biotrypcase (BT) +Thio; M2: BT +Thio+H_2: M3: BT+S; M4: Lactate+SO_4; M5: BT+Thio+O_2; M6: Glucose; M7: BT).

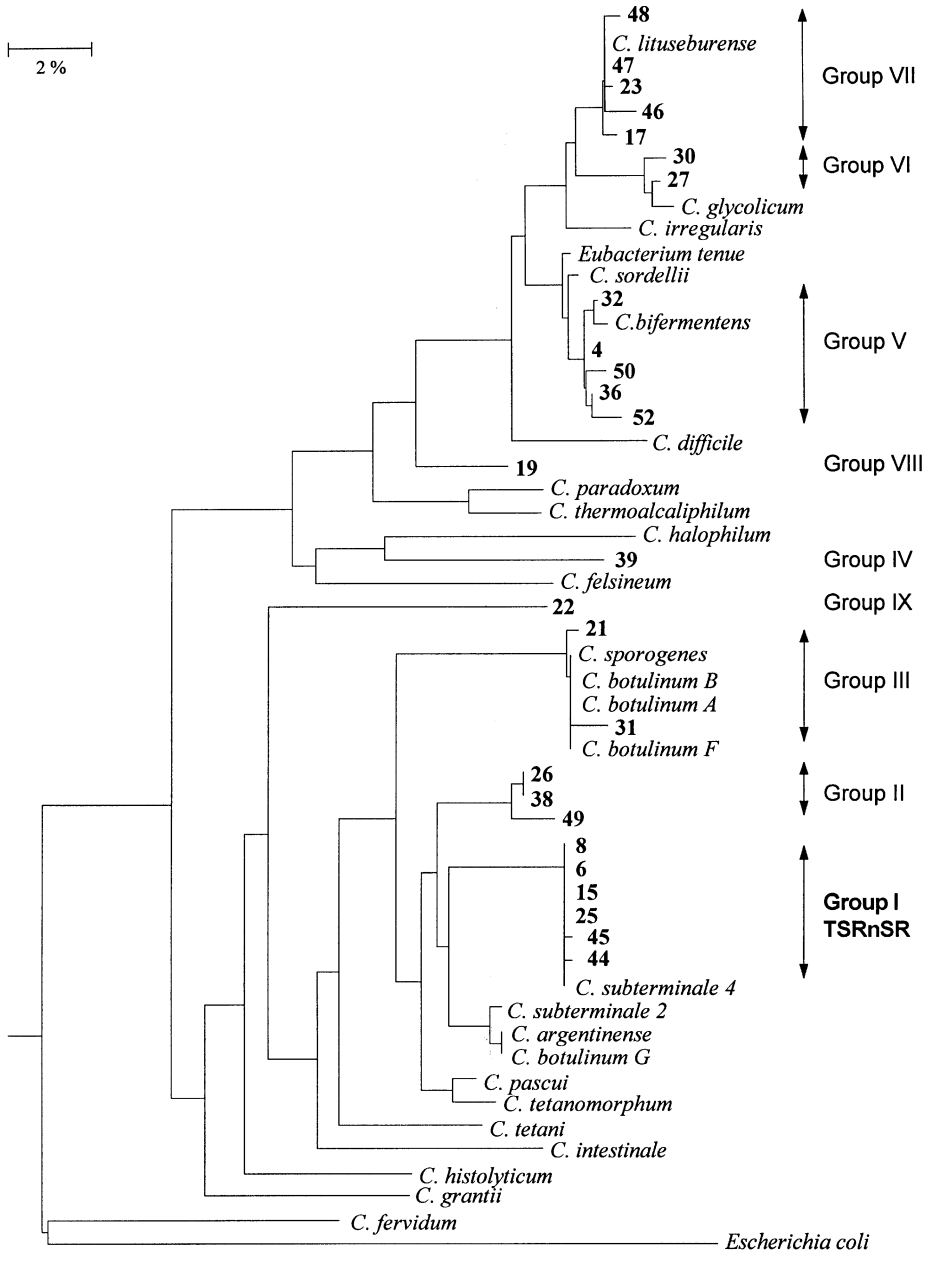
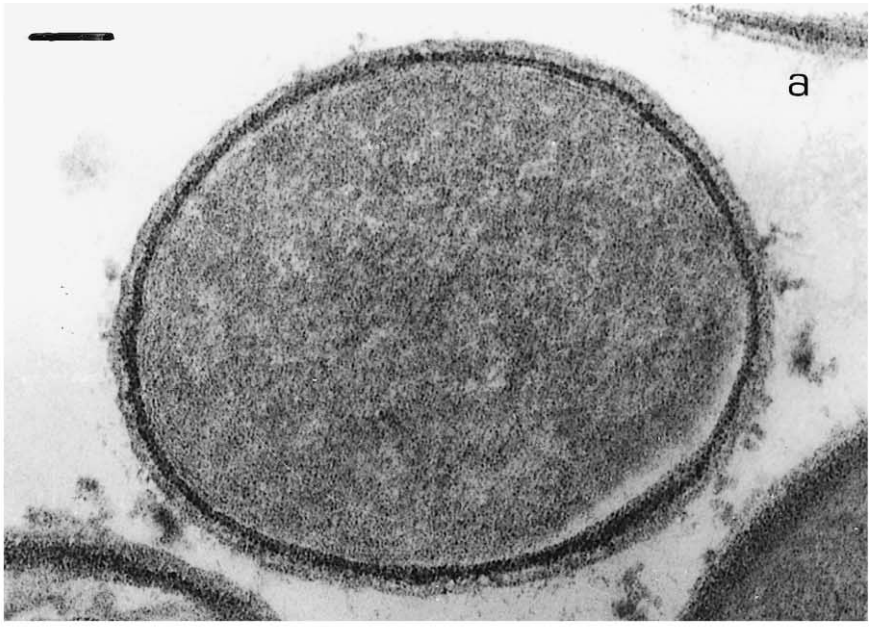


Figure 2. 16S rDNA-based phylogenetic dendrogram showing the position of the strains and their closest relatives, based on 552 unambiguous bases. The scale bar represents two inferred nucleotides changes per 100 nucleotides analyzed.

strain 25 and +15% for strain 45) as observed with other TSRnSR [10]. Growth rates were also higher during the exponential phase of growth in the presence of thiosulfate both in the absence (+36% on average) and the presence (+52%) of H_2. Total H_2S production at the end of the growth phase was significant (4 and 6 mM) in tubes containing thiosulfate and H_2. In tubes containing thiosulfate but no H_2, very little H_2S was produced but growth was much better than in the absence of thiosulfate. Thus thiosulfate was used without S° or H_2S production (if S° had been produced, it would have been reduced to H_2S ; also no S° granule was observed). The metabolic pathway and the nature of the compound(s) resulting from thiosulfate reduction were not elucidated. Species of *Clostridium* and in particular *C. subterminale* ATCC 25774^T produce significant quantities of volatile organosulfur compounds such as methanethiol, dimethyl disulfide, and dimethyl trisulfide [23]. Our experimental procedure did not include the analysis of such compounds.



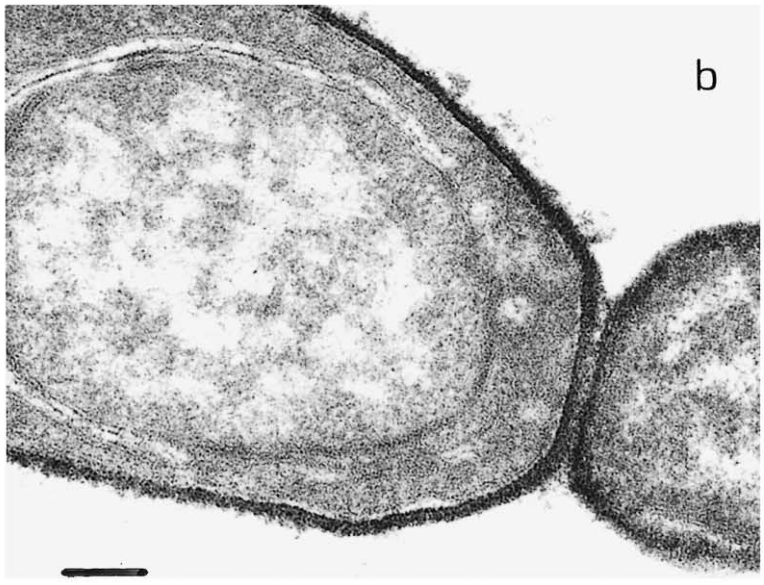


Figure 3. Electron micrograph of a thin section of strains 25 (a) and 45 (b). (bars = 0.5 µm)

3.5.4. Comparison between strains 25 and 45, and C. subterminale

DNA-DNA hybridization between strains 25 and 45 showed 81.5 % similarity, indicating they belonged to the same species [26]. Both strains used the same AA. However, strain 25 used casaminoacids, whereas strain 45 did not. Electron microscopy showed some structural differences in the outer layer of the cell wall, with a fine continuous crenated surface layer surrounding the cell wall in strain 25, which was either discontinuous or not observed in strain 45 (*figures 3a and b*).

We found ambiguities in the deposited sequences of *C. subterminale* 2 and 4, which are both attributed to ATCC 25774 (= DSM 758 according to Bergey's

Table II. Growth of strains 25 and 45 on amino acids (10 mM) with and without 20 mM thiosulfate.

Strain	25		45	
	Thiosulfate			
Amino acids	+	–	+	–
L-Lysine	+	+	+	+
L-Arginine	+	–	+	–
DL-Histidine	+	+	+	+
L-Serine	+	+	+	+
DL-Threonine	+	–	+	–
DL-Tyrosine	–	–	–	–
L- Aspartine	+	–	+	–
L-Gluramine	–	–	–	–
DL -Aspartate	–	–	–	–
L-Glutamate	–	–	–	–
L-Glycine	–	–	–	–
DL -Alanine	+	–	+	–
DL -Valine	+	–	+	–
DL -Leucine	+	–	+	–
L -Isoleucine	+	–	+	–
L -Methionine	+	–	+	–
L -Phenylalanine	–	–	–	–
DL -Proline	–	–	–	–
L -Cysteine	+	–	+	–
DL -Tryptophane	–	–	–	–

manual [6]), but significantly differed from each other (*figure 2*). Therefore, we compared the 16S rDNA of strains 45 and 25 with the sequences of the three available collection strains described as *C. subterminale* (DSM 758, 2636 and 6970^T); Strain DSM 6970^T is cited as type strain in the DSM catalog and as ATCC 25774. It was already sequenced twice (*C. subterminale* 2, X68451; *C. subterminale* 4, L37595). Strain DSM 758 (= ATCC 25774) is cited as type strain in Bergey's Manual (23), where it is used for the phenotypic description of *C. subterminale*, together with 92 other strains. Strains DSM 758 (= ATCC 25774) and DSM 2636 (=ATCC 29748) were not previously sequenced.

The new sequence of DSM 6970^T (AF 241844) showed a satisfactory homology with the sequence of *C. subterminale* 4 (L37595) but markedly differed from that of *C. subterminale* 2 (X68451) which was omitted in further comparisons.

A phylogenic tree including strains 25 and 45 and the strains DSM 758, DSM 2636 and DSM 6970 ^{T} of *C. subterminale*, showed that strain DSM 6970 ^{T} was closest to strains 25 and 45 (0.61 % difference with strain 25 and 0.74 % with strain 45) (*figure 4*).

DNA-DNA hybridization between strain 25 and strain DSM 6970^T showed a similarity of 68.9 %, very close to the theoretical value allowing to define a new species. DNA-DNA hybridization between strain 25 and strains DSM 2636 and DSM 758 showed similarities of 33.8% and 47.2% respectively. Both strains DSM 6970^T and DSM 758 are considered as ATCC 25774^T and as type strains, but clearly differ from each other as indirectly indicated by DNA-DNA hybridization with strain 25, which indicates some taxonomic problem among the deposited strains of *C. subterminale*

Strains 25 and 45 are close to the type strain DSM 6970^T. They therefore belong to the species *C. subterminale*. They differ genetically from strains DSM 2636 and DSM 758, which should be reclassified to another species. Thus, a revision of the taxonomic status of strains and sequences deposited as *C. subterminale* is needed.

The phenotypic comparison of strains 25 and 45 with the description of *C. subterminale* in the Bergey's manual [6], based on strain DSM 758 and 92 other strains is presented thereafter .

Similarly to the description of *C. subterminale*, strains 25 and 45 are straight rods, Gram positive, motile, with the same dimentions ($0.5 - 1.9 \times 1.6 - 11\mu m$). Spores are ovale, most often subterminal, sometimes terminal and distending the cell. Optimal temperature is also quite close (37° for *C. subterminale* and 34°C for strains 25 and 45). However, optimum pH is more alcaline for strains 25 and 45 (8.2) than for *C. subterminale* (6 to 6.4). An optimum pH for growth of 8.2 is in agreement with previous observations showing a positive correlation between soil pH and the abundance of TSRnSR [10]. Metabolic products of *C. subterminale* grown on PYG are mostly acetate, butyrate, isovalerate and to a lower extent isobutyrate [6]. Small quantities of formate, propionate, isocaproate, lactate, succinate, and traces of ethanol and other alcohols can also be produced. The same metabolic products are observed with strains 25 and 45. However, glycine, phenylalanine, tyrosine, and tryptophane are used by *C. subterminale* and not by strains 25 and 45. *Clostridium subterminale* (DSM 758) is slightly more tolerant to NaCl (6.5% required for inhibition) than strains 25 and 45 (5% required for inhibition). Hydrogen production is abundant with *C. subterminale*, whereas it is rarely observed with strains 25 and 45 (1 mM H_2, only when grown on arginine).

. DISCUSSION

In a previous work [10], we provided the first evidence that culturable thiosulfate-reducing bacteria unable to reduce sulfate (TSRnSR) are present in ricefield soils, with an abundance of the same order of magnitude as culturable sulfate reducers using lactate. TSRnSR may therefore be significantly involved in S cycle of wetland soils.

Four soils known or expected to harbour populations of TSRnSR were used to isolate strains. Four enrichment methods were used to vary the selection pressure and increase the chances of isolating a broad range of strains. Among 136 anaerobic non-sulfate reducing mixed cultures isolated on thiosulfate + peptides, 106 reduced S and, among those, 52 reduced thiosulfate, indicating that strict sulfur-reducers (un

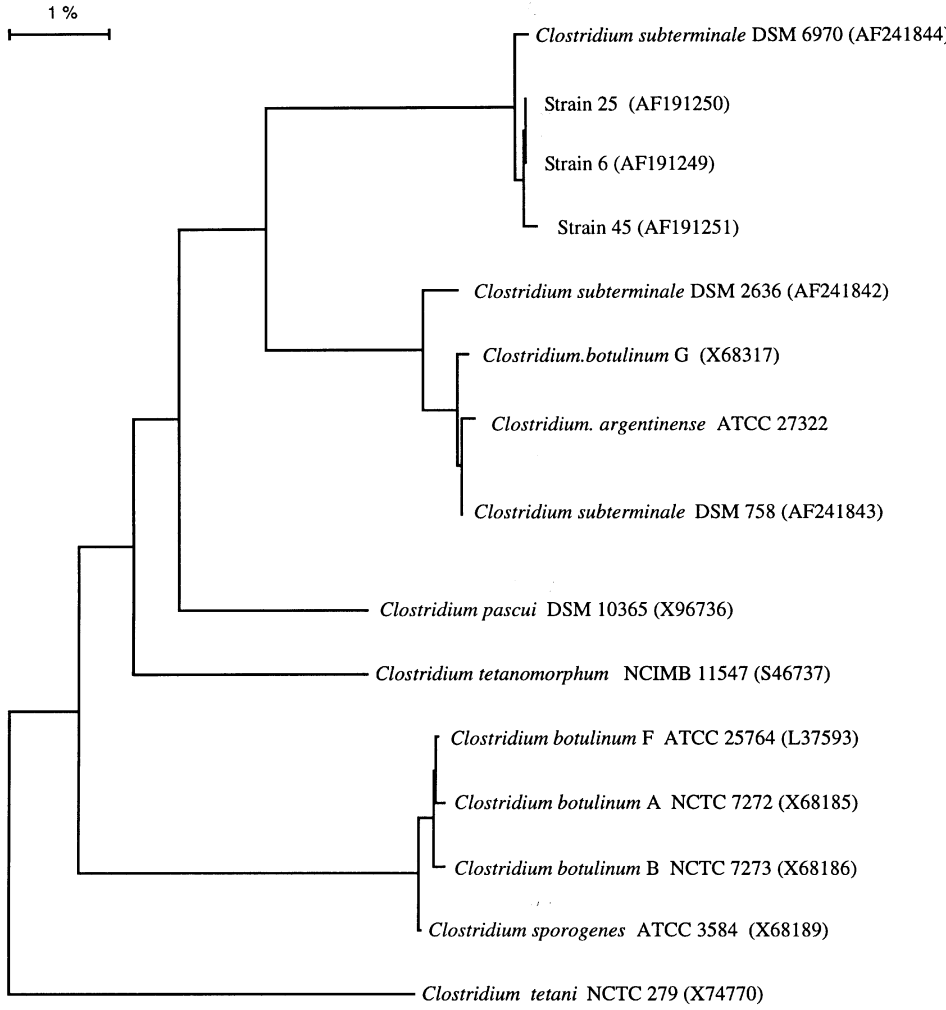


Figure 4. 16S rDNA-based phylogenetic dendrogram showing the position of strains 25 and 45 and their closest relatives, based on 1365 unambiguous bases. The scale bar represents two inferred nucleotides changes per 100 nucleotides analyzed.

able to reduce sulfate and thiosulfate), were probably two-times more abundant than TSRnSR, as culturable organisms, in the four rice soils tested. Among the 52 pure cultures isolated from the 52 TSRnSR mixed cultures, we isolated 18 TSRnSR strains, and 34 strict sulfur-reducers, which can also grow on biotrypcase.

The 18 TSRnSR strains were proteolytic anaerobes unable to use glucose, producing H_2S when grown on thiosulfate + H_2, or when reducing S°. All strains belonged to the same RFLP group comprising only strains of the genus *Clostridium*. Sequencing the 16S rDNA showed that they were phylogenetically close to *C. subterminale*. Data are insufficient to conclude to the ubiquity of this species in ricefield soils, but suggest that it may probably be common.

Phenotypic and genetic information on *C. subterminale* is fragmentary and controversial. Sequencing of the three strains deposited as *C. subterminale* indicates that they might be different species. The deposited strain (DSM 6970^T) closest to strains 25 and 45 has not yet been described phenotypically. The only phenotypic description available for *C. subterminale* is that of strain DSM 758. It shows some differences with strains 25 and 45. Available information clearly shows that a taxonomic revision of *C. subterminale* is needed.

Clostridium is known to be ubiquitous in rice soils. *Clostridium* spp. have been recorded as N_2 fixers in the rhizosphere of cultivated and wild rices [20, 21], polysaccharolytic organisms [7], first colonizers on decomposing rice leaf sheaths [16], and mainly as pesticide degraders. Roger and Bhuiyan [24] in their review on the fate of pesticides in ricefields cited five references reporting the degradation of various insecticides, including chlorinated hydrocarbons, by *Clostridium* strains isolated from ricefield soils.

Previous counts of TSRnSR in ricefield soils [10], their identification as *Clostridium* strains, and the known ubiquity of *Clostridium* spp.in ricefiel soils, indicate that TSRnSR could be important for S cycling in ricefields. Further population dynamic and geochemistry studies are needed to confirm this hypothesis.

Studies aiming at characterizing microbial populations involved in the production of H_2S detrimental to rice were currently testing only sulfate reducing bacteria [13, 25]. Our results suggest that the populations of thiosulfate reducing non-sulfate reducing bacteria should also be considered in such studies.

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