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Structure and Activity of Fe(III)-Reducing Microorganism Occurring in Paddy Fields of Thailand[1a]

Affi Jeanne BONGOUA-DEVISME[2], Aurélie CEBRON[1], Emile Kouadio YOBOUE[2], Christian MUSTIN[1], Jacques BERTHELIN[1]

[1]Laboratory of Interdisciplinary Continental Environments (LIEC), UMR 7360, CNRS (National Center of Scientific Research), Science Faculty, Lorraine-University, B. P. 70239, 54506 Vandoeuvre-les-Nancy Cedex, France

[2]Department of soil Science, UFR STRM, Félix Houphouët Boigny University, Côte d'Ivoire

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Abstract: In the saline paddy soils of Thailand, four bacterial consortia (S1, S2, S6, and S8) were isolated in paddy soils and then selected for their abilities and efficiencies to reduced iron oxyhydroxyde (goethite) at different concentration of salt in culture medium. In this study, the effect of salinity on the structure and activities of bacterial consortia involved in iron reduction process were studied at two saline concentrations (0 and 3% NaCl). The results show that the bacterial consortia presented different behaviors in the presence or absence of salt. The bacterial consortia S1 and S2 were not affected by the presence of salt. Bacterial consortium S6 had a higher iron-reducing activity under saline conditions than in a non-saline environment. The iron reducing activity of the bacterial consortium S8 was inhibited by the presence of salt but not fermentation processes. In this rice fields, the presence of diverse halosensitive to slightly halophilic bacterial groups and also of bacteria presenting both fermentative and iron-respiring metabolisms are able to maintain a strong ability in iron-reduction and dissolution under changing saline environmental conditions.

Keywords: iron-reducing bacteria, Fe(III)-reducers, paddy soil, Thailand, salinity, isolated consortia, bacterial populations

INTRODUCTION

Soil salinity is an increasing problem in agricultural soils throughout the world (***Qadir et al., 2000; Wichern et al., 2006***), and is one of the two main contraints which often affected paddy soils. Soil salinity is caused both by natural phenomena (climate, rock salt deposition and saline groundwater) and human activities (irrigation, drainage, and amendments) (***Emmerich et al., 2012; Williams, 2002***).

Salinity causes osmotic stress which alters the composition and activity of the microbial community and kills sensitive microorganisms (***Ahn et al., 2012; Bongoua-Devisme et al., 2012; Oren, 2011)***. Despite this, soil microorganisms have the ability to adapt to or tolerate osmotic stress caused by drought or salinity, (***Oren, 2001 and 2002; Wichern et al., 2006***). In saline environments, two groups of microorganisms have been distinguished, including the halotolerant and halophile microorganisms (***Youssef et al., 2012***). The halotolerant microorganisms can survive and even grow in relatively high concentrations of salt but prefer to live in the absence of salt (***Kivistö and Karp, 2010)***. Halophiles are microorganisms that require high salt concentrations for growth. Halophilic microorganisms are categorized based upon salt concentration requirements: moderate halophiles can grow at NaCl concentrations of 3 - 15 % (wt/vol) (***Ventosa et al., 1998; Wang et al., 2010***) and extreme halophiles can grow at NaCl concentrations of 15 - 30 % (wt/vol) (***Quillaguamán et al., 2006; Oren, 2011***).

The majority of observations made in saline environments showed a decrease in metabolic processes (***Oren, 2011***). Denitrification, for example,

Affi Jeanne BONGOUA-DEVISME (Correspondence)
bongoua_jeanne@yahoo.fr
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as been observed at 300 g NaCl L^{-1}, while sulfate reduction has been shown to occur up to 240g NaCl L^{-1} (***Sorokin et al., 2010***). However, metabolic processes involved in iron biogeochemical cycling in anaerobic saline environment are scarce. Therefore, an upper salinity limit for microbial Fe(III) reduction has not been defined yet (***Emmerich et al., 2012***).

Microbial populations involved in Fe(III) reduction in saline environment have not been well characterized (***Ahn et al., 2012; Bongoua-Devisme et al., 2012; Emmerich et al., 2012; Pollock et al., 2007; Williamson et al., 2013***). Much works, in which microbial Fe(III) reduction process has been studied, have been performed in non saline environment (***Cummings et al., 2000; Hori et al., 2010; Jiangzhou and Dong, 2008; Li et al., 2011; Lu et al., 2002, 2008***; ***Scala et al., 2006; Wang et al., 2009)***. Microbial iron (III) has been detected in saline environments in up to 125g NaCl L^{-1} or 5 M NaCl (***Emmerich et al., 2012, Bongoua-Devisme et al., 2012, Swan et al., 2010***). Moreover, Alkaliphilic organisms, such as *Bacillus* spp. and *Alkaliphilus metalliredigens*, capable of Fe(III) reduction have been recognized to occur in alkaline environment such as Soap Lake at pH levels as high as 11 and salinity of 125g L^{-1} NaCl (***Gorlenko et al., 2004; Pollock et al., 2007; Ye et al., 2004***). Under these extreme conditions, the presence of a mixed respiratory and fermentative Fe(III) bacterial community could increase the amounts of simple acids (***Markwiese and Colberg, 2000; Pollock et al., 2007***), which would then be available for oxidation by other respiratory metabolisms.

Bacterial species that mediate Fe(III) reduction are phylogenetically and physiologically diverse (***Lehours et al., 2009; Lin et al., 2007***), and include both facultative and obligate anaerobes. They can be classified as fermentative, sulfur-oxidizing, hydrogen-oxidizing or organic-acid-oxidizing (***Jiangzhou and Dong, 2008; Lehours et al., 2009***). Fe(III)-reducing bacteria (FeRB) can be separated into two groups: those that support growth by conserving energy from direct electron transfer to Fe(III) ("Obligate FeRB") and those that use Fe(III) as a supplementary terminal electron acceptor (***Lehours et al., 2009; Lovley et al., 2004***) by fermenting sugars or amino acids and generating acetate, alcohols, H_2 and other fermentation products (***Francis et al., 2000; Lovley, 1991; Ye et al., 2004***) ("Facultative FeRB").

Studies on the Fe(III)-reducing bacteria (FeRB) often consider only specific populations or the activity of the heterotrophic communities (***Lovley and Coates, 2000; Stemmler and Berthelin***, ***2003***). Furthermore, salinity has been studied mainly as a parameter involved in decreasing microorganism proliferation and activity (***Emmerich et al., 2012***; ***Bongoua-Devisme et al., 2012; Oren, 2011)***. In complement of the lack of knowledge on the impact of salinity on FeRB communities structure and activities, Fe(III) reduction seems in Thailand soils, to be involved in the control of pH and particular in the pH increase by proton consumption (***Bongoua-Devisme et al., 2013***) and so to provide better conditions for rice growth in such paddy soils.

Therefore, to better characterize the effect of salinity on the structure of bacterial communities involved in iron-reducing activities, bacterial consortia (S1, S2, S6, and S8) have been selected from a previous study (***Bongoua-Devisme et al., 2012***), for their different abilities and efficiencies to reduce and solubilize Fe(III) to Fe(II) at different levels of added salt (0, 15, 30, 60, and 90 g L^{-1} NaCl) (***Bongoua-Devisme et al., 2012***). Our previous results have shown that all bacterial consortia studied could grow at salt concentrations up to 60 g L^{-1} NaCl (***Bongoua-Devisme et al., 2012***). In this study, to simulate field conditions, we choose 30g L^{-1} as the higher level of added salt to have a maximum salinity corresponding to seawater (3.7 % NaCl) (***Casamayor et al., 2002***). Furthermore, ***Munns*** (***2002)*** and ***Rengasamy (2006)*** reported that soils which present high salt concentrations (i.e., 2 to 15 dS/m or 1 to 10 $g.L^{-1}$ NaCl or 30 to 200 mM NaCl) are considered as saline soils.

The aim of the present study is to assess the impact mainly of salinity on the structure and activities of microbial Fe(III) reducing communities. The activity kinetics and the capacity of bacterial consortia to dissolve iron oxyhydroxides under different level of salinity were studied by monitoring (1) various chemical and biochemical parameters (pH, Eh, glucose consumption, release acids, and iron reduction and solubilization) and (2) the population structures of the bacterial consortia using a fingerprinting method (PCR-TTGE) and sequencing the dominant bacterial members.

MATERIALS AND METHODS

Soils characteristics, sampling and choice of bacterial consortia

Two paddy fields located in Northeast of Thailand at Khon Kaen (16°21'N, 102°36'E) were selected for sampling. One received organic matter (OM) that contained crop residue and manure (paddy field L25), and the other did not receive OM (paddy field L14). The quantity of organic matter addition was estimated at 2.4 t. FW (fresh weight) ha^{-1} ***(Bongoua-Devisme et al., 2012)***. The soils were Ferralsols and classified as Solonchak (salisols) **(*FAO, 1999*)** or as belonging to the Aeric Kandiaquults groups (USDA, classification) (***Grünberger et al., 2008***). Soil samples were collected from the surface horizon (0–20 cm) in November 2003. Each paddy field

contained saline (S) zone with a high soil conductivity > 250 $mS.m^{-1}$ and non saline zone (NS) with a soil conductivity around 150 $mS.m^{-1}$. The four zones in the paddy plots studied (L14NS, L14S, L25NS, L25S) were characterized in a previous study (***Bongoua-Devisme et al., 2012***). Our previous results have shown that Thailand paddy soil samples were sandy (90% of sand particles) and acidic (pH≈5) and contained low amounts of organic matter (0.3 %) and clay (3 to 4%). In these Thailand paddy soils, the total iron (Fe_t) was low (0.22 to 0.23 %) but a large percentage was reducible and available to FeRB: 43 % of total iron were potentially reducible (Fe_{ox} + Fe_{DCB}) by FeRB, 4 % were acid soluble (Fe_{ac}) and 35% were associated with organic matter (Fe_{org}) (***Bongoua-Devisme et al., 2012***). All of these forms (Fe_{ox}, Fe_{DCB}, Fe_{ac}, and Fe_{org}) can be available for bacterial reduction or acid chelating agent production and/or for organic matter biodegradation for the Fe_{org} form. Furthermore, the content of ferrous iron, sulfate, and dissolved organic carbon (DOC) in the soil water collected in the Thailand rice field (piezometers) were respectively 55 to 56 mg L^{-1} of Fe^{2+}, 288 to 960 mg L^{-1} for SO_4^{2-}, and 5 to 6 mg L^{-1} for DOC (***Bongoua-Devisme et al., 2012***), and the fraction of the Fe in these paddy fields occurred in the form of the mineral goethite which was more used in laboratory experiment (***Schwertmann and Cornell, 2000***).

The four bacterial consortia (S1, S2, S6, and S8), used in this study, were isolated from soil samples from a previous study (***Bongoua-Devisme et al., 2012***): S1 - S2 were originated from L25 and S6 - S8 from L14 soils with S1-S6 located in saline zone and S2-S8 in non saline zone.

Bacterial consortia were isolated from soil-dilution (***Bongoua-Devisme et al., 2012***): 4 mL aliquot from the lower dilution of each soil sample-positive well of iron reducing bacteria media was used for selection of bacterial consortia, then was conserved in 1.5 mL of glycerol, stored at -80°C and used in this experiment. These isolates bacterial were selected for their different abilities and efficiencies to reduce and solubilize Fe(III) to Fe(II) at different levels of salt contents. Previous investigations revealed the presence of iron-reducing bacterial communities and their adaptation to salts (***Bongoua-Devisme et al., 2012***). Ours previous results indicated that bacterial consortia S1, S2, and S6 have a high iron-reducing activity in saline conditions at salt concentrations up to 60 g L^{-1} NaCl and consortium S8 was very active at salt concentrations up to 30 g L^{-1} NaCl (***Bongoua-Devisme et al., 2012***). In this study, to simulate field conditions, we choose 30g L^{-1} as the higher level of added salt to have a maximum salinity corresponding to seawater (3.7 % NaCl) (***Casamayor et al., 2002***).

Experimental design of iron oxyhydroxyde dissolution by the bacterial consortia

The culture was performed in flasks (sterilized centrifuge tube, 525-0384 VWR, Strasbourg, France) containing 50 mL of modified medium (BrFe medium): 0.15 g yeast extract (Difco, France), 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, $7H_2O$, 1 g $(NH_4)_2SO_4$, and 0.067 g $CaCO_3$ per liter of ultrapure water, as described by ***Bousserrhine et al. (1999)***. Two different salt concentrations of the medium (0, and 30 g L^{-1} NaCl) were used and the pH was adjusted to 6.5. The medium was sterilized by autoclaving at 110 °C for 30 min as in others studies (***Balch and Wolfe, 1976; Bongoua-Devisme et al., 2012; Schippers and Jørgensen, 2001***). After sterilization, each flask received 0.2 g of D-Glucose (solution at 10 g L^{-1} sterilized separately by filtration at 0.22 µm Millipore filter) and 87.5 mg of iron oxyhydroxyde powder (Prolabo, France), made mainly of poorly crystallized goethite.

To obtain bacterial community of 1 x 10^8 cells g^{-1} dry soil in the medium, 5-mL aliquots of a diluted (10^{-1}) from four isolated bacterial consortia were used to inoculate flasks (sterilized centrifuge tube, 525-0384 VWR, Strasbourg, France) containing 50 mL of modified Bromfield medium. Non-inoculated flasks were used as abiotic control. Three replicates per bacterial consortium and per time of sampling were performed. Flasks were incubated under anoxic conditions in jars gassed with 80% N_2, 10% CO_2, and 10% H_2 after air evacuation by the Anoxomat automatic system MART® WS 80 at 28 °C in the dark for 30 days while stirring. At 2, 4, 8, 15 and 30 days of incubation, the culture suspension was centrifuged at 7 500 r min^{-1} for 10 min at 5 °C. Different parameters, pH, Eh, and contents in ferrous iron, total iron, organic acids, and glucose consumption were determined in the filtered supernatant (0.22 µm). The pellets were frozen at -80° C, stored at -20° C and used for DNA extraction. The pH and Eh were determined using a Tacussel PHN 81 (Choffel Electronic). Ferrous iron was analyzed with the Merck ™ colorimetric kit (Eisen Test 8023) at 523 nm. For the total iron (Fe(II) and Fe(III)), the culture solution was acidified with two drops of HCl (11 N) to prevent iron precipitation, and the analysis were done by inductively coupled plasma atomic emission spectrometry (ICP-AES, Jobin Yvon 2038, France). Organic acids were analyzed by ionic chromatography on an Aminex HPX-87H sulfonic cation-exchange column (300*7.8 mm I.D., BioRad Labs, Marne la Vallée, France). Elution was performed at 50° C with a solution containing 5 mmol.L^{-1} H_2SO_4 for 28 min at a flow rate of 0.5 $mL.min^{-1}$. Organic anions were detected at 210 nm using an UV detector. Glucose was measured by spectrophotometry with the GOD-PAD Kit (bio-Labs, Maizy, France) based on the enzymatic oxidation of glucose in gluconic acid and

the formation of a pink complex measured by optical density at 500 nm.

DNA extraction, 16S rRNA gene amplification and TTGE profiling

Total genomic DNA was extracted from the bacterial pellet collected by centrifugation of the culture suspension. The bacterial pellet received 200 µL of NaCl 0.85 % solution and was pretreated with 20 µL of lysozyme (10 mg mL^{-1}) during 30 min at 37 °C then with 20 µL of Proteinase K (1 %) during 30 min at 70 °C in a water-bath. DNA was extracted using the bead beating protocol described by ***Cébron et al.*** (***2008***). DNA was extracted from triplicate flasks and each replicate was analyzed separately. Bacterial 16S rRNA gene fragments were amplified using the universal primer set 968F-GC/1401R (***Bongoua-Devisme et al., 2013***). The polymerase chain reaction (PCR) was performed in a 50-µL reaction volume containing 1X PCR Buffer (Invitrogen, France), 1.5 mmol L^{-1} $MgCl_2$, 200 µmol L^{-1} of each deoxy-ribonucleoside triphosphate (d'NTP) (Fermentas, France), 0.2 µmol L^{-1} of each primer, 1.25 U of recombinant *Taq* DNA polymerase (Invitrogen, France) and 1 µL of template of DNA. Amplification of 16S rRNA was carried out in an iCycler (Bio-Rad, France) using the following temperature profile: 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 45 s at 56°C, and 45 s at 72 °C; and 7 min at 72 °C. PCR products were analyzed by electrophoresis on a 10 g L^{-1} agarose gel and visualized using a GelDoc transilluminator (Bio-Rad, France) after staining with 0.5 µg mL^{-1} ethidium bromide. Temporal temperature gradient gel electrophoresis (TTGE) was performed using the DCode system (Bio-Rad, France) with a vertical polyacrylamide gel containing 60 mL L^{-1} acrylamide/bis-acrylamide, 7 mol L^{-1} urea, 20 mL L^{-1} glycerol, 1 mL L^{-1} ammonium persulfate, and 1 mL L^{-1} etramethylethylenediamine (TEMED) in 1.25x Tris–acetate-EDTA (TAE) buffer (***Sambrook and Russel, 2001***). The PCR products were run for 5 h at a constant voltage of 100 V, with a temperature gradient from 57 to 67 °C (2 °C increase per hour). After electrophoresis, the acrylamide gel was stained with SYBR Gold (0.1 mL L^{-1}, Molecular Probes, Inc., France) and visualized using a GelDoc transilluminator (Bio-Rad, France) coupled with Quantity One software. The bands of interest were extracted from the gel and reamplified using the same PCR method and the 968F primer without a GC-clamp. The reamplified PCR products were purified using the high pure PCR product purification kit (Roche Diagnostics, France) and sequenced (Eurofins-MWG Biotech, France). Approximately 390 bp of the 16S rRNA partial sequences were aligned using the BioEdit software and compared to 16S rRNA gene sequences obtained from BLASTN in the GenBank database.

Data Treatment and statistical analyses

To express the efficiency of the bacterial consortia to reduce and solubilize iron using mainly glucose as major electron donor for growth and activity, a bacterial iron dissolution index (Id) was calculated and expressed as the percentage of solubilized iron (FeII, and III) released relatively to the total iron added as ferric oxyhydroxide (goethite).

Iron dissolution and glucose consumption kinetic model were first order and defined by $A= A_0 (1-e^{-k\tau})$ for glucose or $A= A_0\ e^{-k\tau}$ for goethite dissolution, with A= amount of glucose or soluble iron available and A_0 = amount of glucose available or soluble iron at initial time; k = constant of glucose consumption or goethite dissolution and τ the time of incubation (2 to 15 days).

Comparison of the Fe(III)-reducing activities of the isolated bacterial consortia at two levels of salt content was performed with the Newman-Keuls test using XLSTAT software at 95% confidence level.

RESULTS

Iron solubilization and reduction from goethite and glucose consumption by bacterial consortia

The reduction and dissolution of the iron oxyhydroxyde (goethite), added to the medium containing glucose as source of carbon and energy with two levels of added salt (0, and 3% NaCl), presented a large diversity of results that depended on the nature of the consortium, and the salt concentration of the medium.

pH and Eh evolution. No significant decrease in pH and Eh was noticed in the control treatments. However, in presence of the four isolated bacterial consortia, a fast decrease redox of potential from + 400 to - 300 mV and of pH from 6.5 to 3.0 (Fig. 1) was observed, indicating both a strong reducing and acidifying activities. Bacterial consortia S1 and S2, originated from L25 amended paddy field, promoted a fast decrease of redox potential from + 400 to - 300 mV but pH decreased more slowly (6.5 to 4.5) (Fig.1). For bacterial consortia S6 and S8, originated from L14 not amended paddy field, both Eh and pH decrease fastly after two days of incubation (Fig. 1).

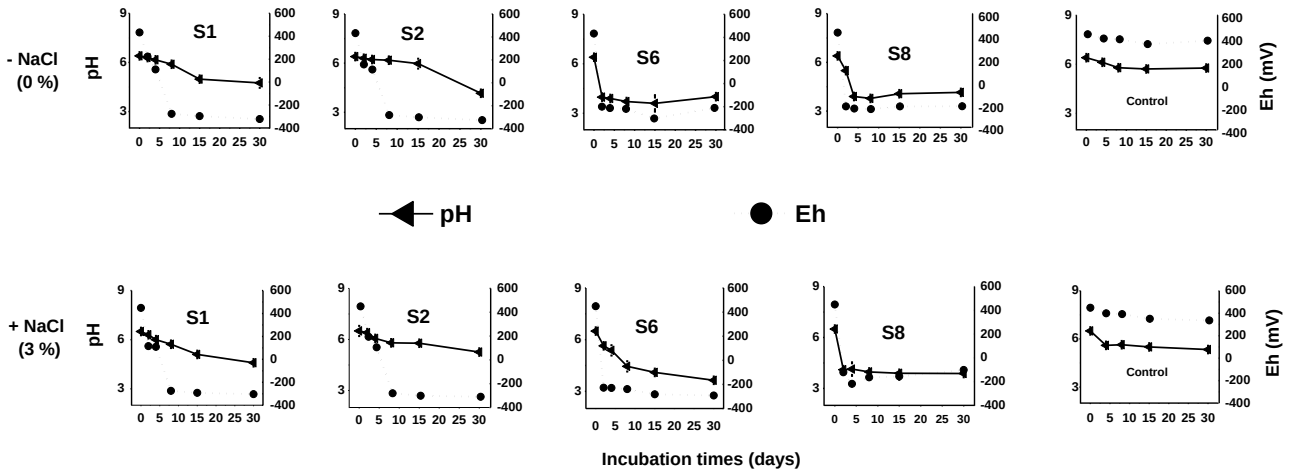

Fig. 1: pH and Eh monitoring in culture medium with or without salt and inoculated by bacterial consortia S1, S2, S6, and S8. Control (abiotic treatment). Mean (n=3) and standard deviation. Dash Dot line = Eh; Solid line=pH

Fe(III) reduction and solubilization from the ferric oxyhydroxyde goethite. The bacterial reduction and dissolution of the iron oxyhydroxyde (goethite), added to the medium, was presented in Figure 2. A large diversity was observed with a reduction and dissolution of iron depending on the nature of the bacterial consortium, and the salt concentration of the medium. No reduction or dissolution of ferric oxyhydroxyde was observed in the control treatments (Fig. 2), underlining the major role of bacterial consortia in the weathering process of ferric oxyhydroxides. In presence of the four isolated bacterial consortia, ferric iron from goethite was mainly solubilized as ferrous iron (Fe^{2+}) but present different behaviors depending on the consortia and of the saline conditions of the medium.

Bacterial consortia S1 and S2, originated from L25 amended paddy field, showed the similar behaviors at 0 and 3 % of salt. Bacterial consortium S1, isolated from the saline zone, was very active in iron reduction and solubilization in both saline and non-saline conditions of medium, after 8 days latence phase. This consortium reduces 50%-70% of total iron in both saline conditions after 15 days of incubation. The bacterial consortium S1 appears not very sensitive to moderate saline condition corresponding to paddy field environment. The calculation of the kinetic constant of iron dissolution from goethite which was similar in presence or absence of salt (Table I) (k = 0.33 and 0.34 respectively) confirms this consortium behavior.

Bacterial consortium S2, originated from the non-saline zone dissolved as more rapidly as S1 the goethite in the absence of salt (k = 0.35) but has a lower rate under saline conditions (k = 0.22), with 15 days of latence phase. For S2, 20%-25% of total iron was reduced and solubilized in medium containing 30 g L^{-1} NaCl, after 15 days of incubation, while 50%-70% of total iron in medium without salt.

Bacterial consortia S6 and S8, originated from L14 not amended paddy field, present different behaviors in presence and absence of salt in the medium. Bacterial consortium S6, originated from the saline zone dissolved more rapidly the ferric oxyhydroxide in saline conditions (k = 0.31), with 50%-75% of total iron reduced after 15 days of incubation than in absence of salt (Fig. 2; Table I) (k = 0.22), reducing 20%-25% of total iron. For bacterial consortium S8, originated from non saline zone, the goethite was rapidly dissolved in absence of salt during the first 8 days of incubation (k = 0.76), with 55%-75% of total iron reduced, whereas in saline conditions the dissolution was very low (k = 0.11), reducing 0%-1% of total iron.

Glucose consumption. Simultaneously to the absence of iron reduction and dissolution in control treatments no detectable glucose consumption was observed (Fig. 2). Bacterial consortia S1 and S2, originated from L25, were very active in consumption of glucose in both saline and non saline conditions after 8 days latence phase (Fig. 2). However, for bacterial consortia S6 and S8, isolated from original paddy soils (L14), the kinetic of glucose consumption was different. Bacterial consortium S6 used fastly glucose in both saline conditions but for bacterial consortium S8, glucose was almost totally consumed in presence of salt while there was very low iron reducing activity (only 2 % of iron was reduced and solubilized).

Table I: Kinetics of glucose consumption and iron dissolution during 2 to 15 days of incubation. $k=1/\tau$ (day $^{-1}$), τ: time characteristic. First order reaction with p>5%.

bacterial consortium	Kinetics of glucose consumption		Kinetics of iron dissolution	
	0% NaCl	3% NaCl	0% NaCl	3% NaCl
	k	k	k	k
S1	0.151 ± 0.01	0.139 ± 0.01	0.33 ± 0.02	0.34 ± 0.02
S2	0.091 ± 0.01	0.091 ± 0.01	0.35 ± 0.01	0.22 ± 0.03
S6	0.107 ± 0.01	0.125 ± 0.01	0.22 ± 0.01	0.31 ± 0.05
S8	0.317 ± 0.02	0.151 ± 0.01	0.76 ± 0.20	0.11 ± 0.03

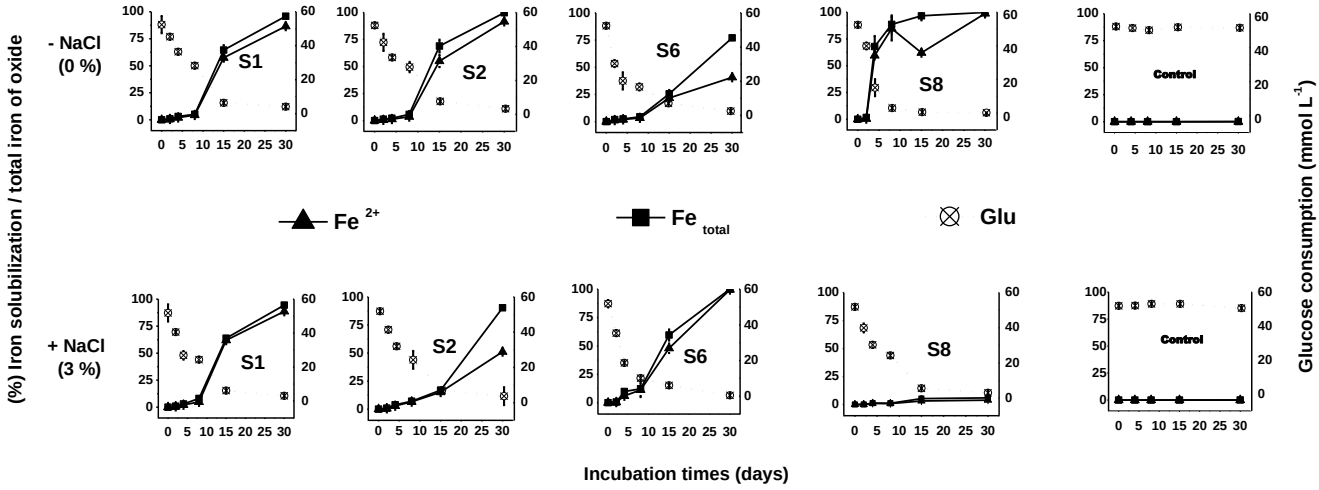

Fig.2: Iron solubilization (as total iron Fe_{total}, and reduced iron Fe^{2+}) and glucose consumption by different bacterial consortia S1, S2, S6, S8 in culture media with or without salt. Control (abiotic treatment). Mean (n=3) and standard deviation. Dot line = glucose consumption; Solid line= iron solubilization

Bacterial release of acids and fermentation occurrence. Various organic acids were released in the culture medium during the incubation simultaneously with reduction processes. Gluconic, succinic, lactic, acetic, propionic, and butyric acids were the main compounds released; indicating fermentation processes (Table II). For bacterial consortia (S1 and S2), originated from L25 paddy field, gluconic, succinic, acetic and lactic acids are produced in both conditions. However, bacterial consortium S2, originated from the saline zone of L25, releases also butyric and propionic acids with an accumulation of butyric acid at low concentration. The presence of salt in medium seems to promote lactic and acetic acids mainly at the end of incubation time (Table II). So in complement of lactic and mixed acid fermentations, butyric fermentation occurred for bacterial consortium S2.

Bacterial consortia S6 and S8, originated from not amended paddy field, release in the medium much more acids (succinic, lactic, acetic, propionic, and butyric) with high amounts than those originated from amended paddy field. For Bacterial consortium S6, lactic occurs largely at 3% salt added while succinic, acetic and propionic acids are present much more in absence of salt. Again lactic, mixed acid, and also propionic acid fermentation seem to be involved. For bacterial consortium S8, the gluconic, succinic, lactic, acetic, propionic and butyric acids were released. Succinic, and acetic acids were detected in high amounts in absence of salt in medium but butyric acids occurred only in presence of salt in the medium. Different types of bacterial

fermentations occurred, depending on saline conditions and time of incubation. Some of these acids can promote other fermentations, e.g. succinic, lactic can be at the origin of propionic acid (Table II).

Behavior of the 4 bacterial consortia appears significantly different in the iron reduction and dissolution processes and organic acids production, depending to the difference of original paddy soils which were isolated the different bacterial consortia.

Table II: Organic acids released (mg L^{-1}) by bacterial consortia (S1; S2; S6; S8)

Table IIa: by S1 originated from the saline zone of L25 amended paddy soil

Consortium S1	time (days)	organic acids					
		gluconic	succinic	lactic	acetic	propionic	butyric
non saline (0% NaCl)	2	116.1	84.1	14.3	17	0	0
	4	57	41.1	23.2	0	0	0
	15	100.4	67.1	721.7	155.5	0	0
	30	121.4	68.1	744.1	219.7	0	0
saline (3 % NaCl)	2	268.5	177.4	105.8	135.7	0	0
	4	286.5	161	40.4	33.8	0	0
	15	130	174.1	3256.7	14.6	0	0
	30	17	139.9	521.9	1517.5	0	0

Table IIb: S2 originated from the non-saline zone of L25 amended paddy soil

Consortium S2	time (days)	organic acids					
		gluconic	succinic	lactic	acetic	propionic	butyric
non saline (0% NaCl)	2	7.0	90.3	41.2	11.2	0	0
	4	133.5	43.0	18.0	2.9	0	0
	15	42.6	23.7	164.2	263.3	32.1	1
	30	20.9	15.1	21.0	60.5	26.1	1.3
saline (3 % NaCl)	2	43.8	1.3	3.6	18.3	23.9	0
	4	42.1	6.5	4.3	0	22.7	1.5
	15	180.6	157.2	232.2	1072	0	1.8
	30	119.1	87.6	222.1	1245.4	0	0

Table IIc: S6 originated from the saline zone of L14 not amended paddy soil

Consortium S6	time (days)	organic acids					
		gluconic	succinic	lactic	acetic	propionic	butyric
non saline (0% NaCl)	2	80.6	1058.7	305.7	172.2	407.5	0
	4	0	1747.8	18.3	1743.7	364.5	0
	15	4.4	1149.4	0	1912.7	339.3	0
	30	46.3	1556.5	58.8	1445.7	263.1	0
saline (3% NaCl)	2	219.3	625.6	1427.6	201.9	451.2	0
	4	109.3	883.5	1717.7	320.5	222.9	0
	15	28.9	434.8	1807.9	22.3	131.3	0
	30	57.2	595.5	1720.6	51.5	105.8	0

Table IId: S8 originated from the non-saline zone of L14 not amended paddy soil

Consortium S8	time (days)	organic acids					
		gluconic	succinic	lactic	acetic	propionic	butyric
non saline (0% NaCl)	2	145.3	65.6	61.1	150.9	0	0
	4	51.9	333.1	55.9	2584	45.9	0
	15	0	1098.9	3.4	3406.4	66.5	0
	30	0	2475.9	29.5	4509	148.3	0
saline (3 % NaCl)	2	52.6	96.2	101.7	41.7	36.6	31.9
	4	25.6	11.2	23.4	132.1	6.5	97.4
	15	27.1	12.3	4.2	384.4	27.4	342.6
	30	25.7	24.8	19.6	106.6	19.3	393.2

Population dynamic in the bacterial consortia during incubation

The TTGE profiles of the 16S rRNA genes showed differences in the bacterial consortia structure and their evolution during incubation times, depending on the salinity of the medium, the carbon source and the time of incubation (2, 4, 8, 15, and 30 days) (Fig. 3). Only one replicate is presented in Fig. 3 for clarity, but the three replicates for the same sampling time of one consortium have similar TTGE profile (data not shown), indicating the reproducibility of the experiments.

The TTGE profiles of the consortium S1 were the same in saline and non saline conditions with the identification of 4 main bacterial species, corresponding to the bands a, b, c and d (Fig. 3). However, a succession of species appeared during the incubation time course. Two bacterial species were always present throughout the incubation time. They were closely related to *Shewanella* sp. and *Shewanella putrefaciens* (bands (b) and (c), respectively). The sequence affiliated with *Clostridium acetobutylicum* (band a) became more dominant after 4 and 8 days in non-saline and saline conditions, respectively. It occurs when strict anaerobic conditions were present. No byturic acid was detected but acetic can be produced by such bacteria. A band (d) appeared after 15 days in saline and non-saline conditions, and the sequence was closely related to the halotolerant and alkaliphilic, *Oceanobacillus iheyensis*. For the bacterial consortium S2 in saline and non-saline conditions, TTGE profiles showed the presence of common bands (1 and 2) that were shown after sequencing to belong to the same species as the S1 consortium (two bacteria closely related to *Shewanella* sp. and *Shewanella putrefaciens*). The consortium was composed of other species corresponding to bands 3 and 4, closely related to facultative anaerobic *Virgibacillus* sp. and *Paenibacillus* sp., which were favored and became dominant after 15 days of incubation in non-saline and saline conditions, respectively. The characterization of the population structure of the bacterial consortium S6 showed the presence of common species of saline and non-saline environments, as bands x, y, and z which were related to *Enterobacter* sp., *Pantoea agglomerans* and *Shewanella putrefaciens*, respectively.

Finally, the TTGE profile of the bacterial consortium S8 showed the presence of 3 common species of saline and non-saline environments, with bands 6, 7 and 8 (Fig. 3) related *Clostridium beijerinckii*, *Clostridium* sp., and *Alkaliphilus metalliredigens*, respectively and the development of *Bacillus spp*. (band 5) in saline condition. In non-saline conditions, bands 6 and 7 seemed less intense, suggesting that the corresponding bacteria were probably less abundant. In all these consortia, there are fermentative bacteria which can be at the origin of the different acid production found in the media.

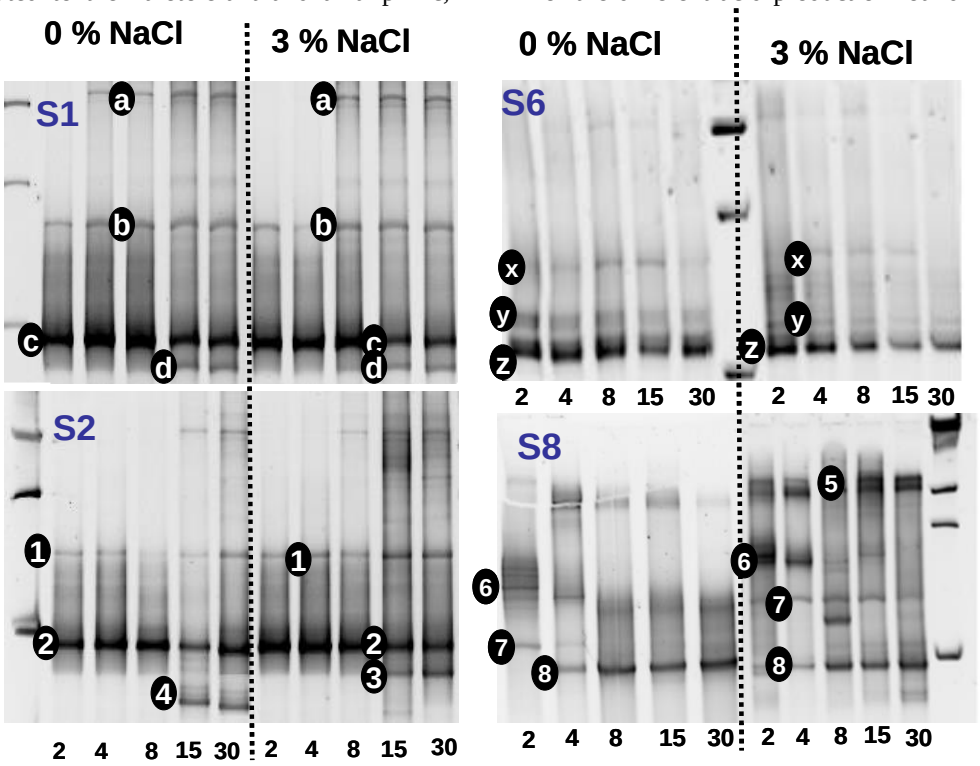

Fig.3 *:* TTGE of 16S rRNA PCR products from 4 bacterial consortia (S1, S2, S6 and S8). Community structure analyses were performed during 30-day of incubation in the presence or absence of salt (3 or 0 % NaCl). The TTGE profiles were similar for the three replicates of the incubation treatments. Only one TTGE profile is shown for clarity. Letters or numbers indicate the different bands that were sequenced for taxonomic identification. Bands:

a = *Clostridium acetobutylicum; b =Shewanella sp., c = Shewanella putrefaciens; d = Oceanobacillus iheyensis* for the bacterial consortium S1. Bands: 1 = *Shewanella sp., 2 = Shewanella putrefaciens; 3 = Virgibacillus sp.; 4 = Paenibacillus sp.* for the S2 bacterial consortium. Bands: x = *Enterobacter sp., y = Pantoea agglomerans, z = Shewanella putrefaciens* for bacterial consortium S6. *Bands: 5 = Bacillus sp., 6= Clostridium beijerinckii, 7= Clostridium sp., 8= Alkaliphilus metalliredigens* for bacterial consortium S8.

DISCUSSION

Ferric iron can be reduced by Fe(III)-reducing bacteria through respiratory (electron acceptor) and fermentation (electron sink) processes (***Lehours et al., 2009)***. A wide diversity of Fe(III)-reducing activities and bacterial populations has been studied in non saline anoxic environments (***Berthelin et al, 2006***; ***Ehrlich, 2002; Lehours et al., 2009; Lin et al., 2007; Lovley et al., 2004; Scala et al., 2006***). However, iron reduction in saline environments, which covered large areas in the world, such as in paddy soils, has not been well documented (***Ahn et al., 2012; Bongoua-Devisme et al., 2012; Emmerich et al., 2012***; ***Oren, 2011)***. Little is known about the halotolerant and halophilic Fe(III)-reducing bacteria which are of major interest of the functioning in such environments. One reason for the interest in halophilic Fe(III)-reducing bacteria is the need to understand the biochemical activities (reduction, acidification processes, release of iron) and highlight the diversity of bacterial communities and their behaviors in saline and non saline conditions ***(Oren, 2011)***. In our study on the impact of salinity on the Fe(III)-reducers activities in paddy fields, the different isolated bacterial consortia present different efficiencies in their ability to reduce and dissolve iron from ferric oxyhydroxides (goethite) and to produce organic acids, depending on the environmental conditions and the nature of bacterial communities. The four studied consortia have different main strains populations which expressed different iron reducing activities depending on salinity of medium (Fig. 3). Kinetic of bacterial iron reduction (k) were 0.11 to 0.76 for S8 consortium indicating a large range of efficiency. S8 had better reduction and dissolution activity in non saline condition than in saline condition. Some consortia are efficient in saline condition (S1, S2, and S6). The bacterial consortia were either stable or variable during the incubation period and regarding saline or non-saline conditions.

Fe(III)-reducers diversity in saline paddy soils fields

The consortia were composed of bacteria belonging to Proteobacteria and Firmicutes. Fermentative Fe(III)-reducers (e.g., *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium* sp., *Paenibacillus* sp., *Bacillus* sp., and *Enterobacter* sp.; ***Dobbin et al., 1999; Lehours et al., 2009; Li et al., 2011; Pollock et al., 2007***) and well-known obligatory Fe(III)-reducers (e.g., *Shewanella putrefaciens, Shewanella* sp. and *Alkaliphilus metalliredigens;* ***Bongoua-Devisme et al., 2012; Li et al, 2011***) were retrieved in the consortia. However, we have also identified marine microorganisms (e.g., *Oceanobacillus iheyensis*, and *Virgibacillus* spp.), that were not related to known obligatory Fe(III)-reducing bacteria or fermentative Fe(III)-reducing bacteria but which are halotolerant ***(Bongoua-Devisme et al., 2012; Lu et al., 2001)*** .

The Fe(III)-reducing bacteria isolated in the studied bacterial consortia, belonging to *Firmicutes* and *Proteobacteria*, , might also be important groups of Fe(III)-reducers in paddy soils. In the isolated bacterial consortia (S1, and S2), originated from amended paddy soil, the bacteria from both phyla group have been observed, while, in bacterial consortia (S6, and S8), originated from not amended paddy soil, the sequence were affiliated with only one group (*Proteobacteria* for S6 and *Firmicutes* for S8), as observed in previous studies (***Ahn et al., 2012 and Williamson et al., 2013***), in which different phylogenetic group and communities have been identified in rice field subjected to fertilization.

Bacterial populations identified from the TTGE profiles revealed a great diversity of bacteria with different metabolic pathways: fermentative (*Bacillus* spp., *Clostridium* spp., *Enterobacter* spp.*;* ***Lehours et al. 2009; Pollock et al. 2007***), iron-respiring (*Shewanella* spp. and *Alkaliphilus metallireducens*; ***Li et al., 2011***) and marine microorganisms *(Virgibacillus* spp. and *Paenibacillus* spp.; ***Jiang et al., 2007***). All the identified bacterium strains have already been observed in saline environment (***Emmerich et al., 2012; Williamson et al., 2013; Sorokin et al., 2010; Swan et al, 2010; Youssef et al. 2012***). In this study, the addition of salt in culture medium modified only the structure of TTGE profile of bacterial consortium S8, with the development of *Bacillus* spp. when salt was added in medium. Therefore, the wide diversity of anaerobic Fe(III)-reducers in this paddy fields seems modified their metabolic functions and their specific activities at high salinities.

Activity of Fe(III)-reducers in saline paddy soil

The composition of bacterial consortia S1 and S2 presented a wide diversity of bacterial strains with the same TTGE profile in the presence and absence of salt throughout the incubation time. For the bacterial consortia S1 and S2 the dissolution of iron oxyhydroxide (goethite) presented the same evolution in saline and non-saline conditions. The high iron solubilization and the absence of variation in the iron reduction kinetics observed may be due to

the activity of different bacterial strains, such as Clostridium spp.; *Paenibacillus* spp. and *Shewanella* spp., identified in the consortia but also to other non detectable bacteria at the origin of fermentation products. In fact, fermentative bacteria (e.g., *Clostridium acetobutylicum; Paenibacillus* spp), can initially produce fermentable substrates (e.g., glucose) and then Fe(III)-respiring bacteria (e.g., *Shewanella* sp.) oxidize the fermented products (e.g., organic acids) and can indirectly reduce iron, as demonstrated in other studies (***Emmerich et al., 2012; He et al, 2011; Lu et al., 2008; Markwiese and Colberg, 2000***). Furthermore, the presence of a mixed population including respiratory and fermentative bacteria in paddy soils plays an important role in global carbon and iron cycling at high salinities (***Emmerich et al., 2012; Islam et al., 2011; Pollock et al., 2007; Valencia-Cantero et al., 2007***).

The characterization of the bacterial consortium S6 shows the presence of the same bacterial species (*Shewanella putrefaciens*, *Pantoea agglomerans*, and *Enterobacter* spp.) in both condition of salinity. The bacterial species identified have already been observed in saline environments (***Stapleton et al., 2005; Bongoua-Devisme et al., 2012***) and can also reduce and use H_2 or simple soluble substrates, such as acetate as an electron donor and ferric oxyhydroxyde as an electron acceptor ***(Francis et al., 2000; Roh et al., 2006; Xiao et al., 2007)***. In this study, after 15 days of incubation, Bacterial consortium S6 reduced and solubilized iron oxyhydroxyde efficiently and more rapidly in presence of salt, with 50%-70% of total iron reduced while 20%-25% in medium without salt. The iron-reducing activities of isolated bacterial consortium S6 revealed that S6 appears not sensitive to presence of salt in the medium and had halotolerant and halophilic characteristics. These Thailand paddy fields contained rich Fe(III)-reducing bacterial populations (*Shewanella putrefaciens*, *Pantoea agglomerans*, and *Enterobacter* spp.) which their specific activities appeared unaffected by salt concentrations under anaerobic conditions (***Stapleton et al., 2005; Slobodkin et al., 1999; Sorokin et al., 2010)***. The activity of these different Fe(III)–reducers favored iron reduction and solubilization at high salinities and can contribute to the amelioration of the quality of soils and water.

For bacterial consortium S8, the presence of salt affects its activity and structure. Here, the iron-reducing activity of the S8 consortium was almost totally inhibited in saline condition in spite of the presence of the well known Fe(III)-reducing bacterial strains, such as *Clostridium* spp., *Bacillus* spp. and *Alkaliphilus metalliredigens*. (***Li et al., 2011; Pollock et al., 2007; Ye et al., 2004***). These results indicated that the presence of salt favored the growth of *Bacillus* spp. but inhibited its biological activity, as previously shown in others studies (***Hoffmann et al., 2002*** and ***Arnold et al., 1990***). In our study, even if *Alkaliphilus metalliredigens* was present at acid pH ($6 < pH < 3$), its iron-reducing activity was totally inhibited by acid conditions. These results are different from those observed by ***Ye et al.*** (***2004***) who indicated the growth of *Alkaliphilus metalliredigens* at pH from 7.5 to 11. The biological activity of *Clostridium* spp. involved in iron cycle by reduction process appeared to be affected by salt concentration. Despite the efficient growth of the bacteria (e.g., consumption of glucose), this assemblage of bacteria in the consortia was unable to solubilized and reduced iron under saline conditions. The S8 consortium can be considered as halosensitive for its iron-reduction activity but halotolerant for some fermentation pathways. As deduced from the organic acid analyses, the difference between saline and non-saline conditions can be related to the expression of butyric fermentation or butyric pathways and acetic fermentation or acetic pathways, respectively. The release of electrons during fermentation, which could be use in Fe(III) reduction, seems to be inefficient. The metabolic pathways of consortia such as S8 have to be better understood to deduce the mechanisms controlling separately or simultaneously the fermentation pathways and the iron-reduction in saline and non-saline conditions.

Iron-reducing kinetics and organic acids production of bacterial consortia originated from amended paddy soil (S1 and S2) were different than those originated from not amended paddy field (S6 and S8). The presence of salt has not significant effect on iron reducing activity of S1, and S2 (halotolerant), but a significant negative effect with S8 (halosensitive) and positive with S6 (halophile). Then, the bacterial consortia located in saline zone of paddy fields, S1 and S6 were not affected by the presence of salt in the culture medium and reduced and solubilized more rapidly iron after 15 days of incubation than those isolated in non-saline zone (S2 and S8). These adaptability of Fe(III)-reducers bacterial in these rice fields of Thailand could be attributed to the presence of diverse halosensitive to slightly halophilic bacterial groups and also to the diversity of bacteria presenting both fermentative and iron-respiring metabolisms, as previously shown in other studies (***Islam et al., 2011***). The presence of anaerobic microbial Fe(III)-reducing suggests an active microbial Fe cycling in Thai paddy soils.

In summary, most of the species identified in the four isolated and cultivated consortia have already been studied in pure culture but not in saline vs non-saline conditions as in Thaï paddy fields. The interactions between iron-reduction and high and low saline conditions were not demonstrated prior this study. The association of various bacteria in such consortia and their different abilities to be active in

saline and/or non-saline conditions allow the bacterial populations to maintain iron-reducing activity even when the environmental conditions are changing. The results highlighted a complementarily presence and activities of species for fermentation and iron reduction and dissolution in saline and non-saline conditions. This study also showed the adaptability of iron-reducing bacterial communities under different conditions of salinity, demonstrating that in paddy field soils these bacteria could be involved in iron reducing activity under both moderate saline and non-saline conditions.

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