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Dissimilatory Fe(III) reduction characteristics of paddy soil extract cultures treated with glucose or fatty acids

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Abstract

Dissimilatory Fe(III) reduction is a universal process with irreplaceable biological and environmental importance in anoxic environments. Our knowledge about Fe(III) reduction predominantly comes from pure cultures of dissimilatory Fe(III) reducing bacteria (DFRB). The objective of this study was to compare the effects of glucose and a selection of short organic acids (citrate, succinate, pyruvate, propionate, acetate, and formate) on Fe(III) reduction via the anaerobic culture of three paddy soil solutions with $Fe(OH)_3$ as the sole electron acceptor. The results showed significant differences in Fe(III) reduction among the three paddy soil solutions and substrate types. Bacteria from the Sichuan paddy soil responded quickly to substrate supply and showed higher Fe(III) reducing activity than the other two soil types. Bacteria in the Jiangxi paddy soil culture solution could not use propionate as a source of electrons for Fe(III) reduction. Similarly, bacteria in the Jilin paddy soil culture could not use succinate effectively. Pyruvate was readily used by cultures from all three paddy soil solutions, thus indicating that there were some similarities in substrate utilization by bacteria for Fe(III) reduction. The use of glucose and citrate as substrate for dissimilatory Fe(III) reduction indicates important ecological implications for this type of anoxic respiration.

Key words: dissimilatory Fe(III) reduction; glucose; organic acid; anaerobic incubation

Introduction

Since the first report of Fe(III) reducing bacteria, which could utilize Fe(III) as the terminal electron acceptor and gain energy from the complete oxidation of organic compounds (Lovley and Coates, 2000), more and more attention has been focused on the process of dissimilatory iron reduction in anaerobic environments. This interest is owing to the important implications that dissimilatory iron reduction can have on nutrient release, biogeochemical transformation of elements, and environmental bioremediation (Lovley and Coates, 2000; Lovley *et al*., 2004).

Thus far, diverse dissimilatory Fe(III) reducing bacteria (DFRB) have been isolated and cultured from various environments (Lovley *et al.*, 2004; Shelobolina *et al*., 2004, 2007). Pure culture studies have shown that DFRB can use a diverse array of substrates as the preferential carbon source, including carbohydrates such as sucrose, maltose, glucose, and xylose, and short organic acids such as lactate, acetate, pyruvate propionate, succinate, malate, and fumarate (Cummings *et al*., 2000; Finneran *et al*., 2003; Roh *et al*., 2002). However, there seem to be differences in carbon use patterns among different types of DFRB. Acetate, for an instance, can be used by a majority of DFRB and often serves as a common substrate for the isolation of DFRB. In contrast, *Acidiphilium cryptum* JF-5 was very sensitive to acetate, showing inhibition of respiration at acetate concentrations as low as 300 μmol/L. Furthermore, acetate (0.3–5.0 mmol/L) also suppressed the Fe(III) reducing activity of this bacteria, though it was able to use glucose, ethanol, or H_2 as substrate (Küsel *et al*., 1999).

Our knowledge about DFRB depends mainly on pure culture studies, which do not reflect natural environment conditions. Previous paddy slurry incubations found that the occurrence and intensity of Fe(III) reduction were very stable even if the soils were dried and then rehydrated before incubation, suggesting that a stable microbial community responsible for Fe(III) reduction may be present and may survive in dried soils (Qu *et al*., 2005a, 2005b). However, our understanding about these microbial communities is very limited. In this study, we investigated the dissimilatory Fe(III) reduction characteristics in three paddy soil extract cultures using glucose and short organic acids as substrates to characterize substrate metabolism under Fe(III) respiration.

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1 Materials and methods

1.1 Inocula preparation

For the preparation of inocula, three paddy soil samples were collected from Anfu County, Jiangxi Province (JX); Gonglai County, Sichuan Province (SC); and Fengman County, Jilin Province (JL), China. The soils were air-dried, ground, and passed through a 1-mm sieve. The soil solutions were prepared by mixing the soil with deionized water at 1:9 (*W/V*). The solutions were incubated in the dark at 25°C for a week to activate the Fe(III) reducing microbial community, and then the soil solutions were shaken and centrifuged at 700 r/min for 10 min. The supernatants were used as inocula in the incubations.

1.2 Anaerobic incubation

Anaerobic incubations were performed in 10 ml serum bottles containing 2 ml basic culture medium, 1 ml ferrihydrite solution, 1 ml of carbon source solution, and 1 ml of inocula. The basic culture medium consisted of phosphate buffer (0.49 g K_2HPO_4/L, 0.23 g KH_2PO_4/L, pH 7.0), 1.0 g NH_4Cl/L, and 1.96 g KCl/L. Ferrihydrite (13.025 g/L) was the sole electron acceptor in the medium. Seven carbon source solutions (20 mmol/L) were compared: glucose, citrate, succinate, pyruvate, propionate, acetate, and formate. The basic culture medium and ferrihydrite solution were autoclaved before being added into serum bottles. The carbon source solutions were passed through a 0.22-μm filter.

The solutions and inocula were added aseptically to the oven dried serum bottles, then, the serum bottles were covered with rubber stoppers, purged with N_2 for 5 min to remove O_2, and sealed with aluminum covers. The serum bottles were incubated in a controlled environment incubator at 25°C.

Three bottles were randomly selected from each treatment at day 0, 1, 3, 5, 7, 10, 13, 16, 19, 22, 27, 32, 42 of the incubation. Each bottle was shaken vigorously to homogenate and then a 0.6-ml aliquot was removed from each culture and transferred to a screw top plastic tube containing 4.4 ml of 0.5 mol/L HCl. The samples were extracted for 24 h at 25°C and then passed through a 0.22-μm filter. Ferrous iron in the filtrate was determined using the ferron colorimetric method (Qu *et al*., 2005a; Schnell *et al*., 1998). Statistic analysis of all the data was conducted by SPSS 13.0. Curve simulations were completed with CurveExpert 1.3.

1.3 Dynamics models

Logistic and exponential models were used to simulate the relationship between Fe(II) accumulation and incubation time in each treatment. The logistic model can be expressed as:

$$C_t = \frac{a}{1 + b \times e^{-ct}} \quad (1)$$

where, C_t is the ferrous iron concentration measured after incubation for t (d), a, b, and c are the parameters in this formula. When C_t is differentiated by t, the reaction velocity is obtained.

$$V = \frac{dC_t}{dt} = C_t \times c \times (1 - \frac{C_t}{a}) \quad (2)$$

The initial derivative of V is:

$$\frac{d}{dt}\left(\frac{dC_t}{dt}\right) = C_t \times c^2 \times (1 - \frac{3C_t}{a} + \frac{2C_t^2}{a^2}) \quad (3)$$

When the initial derivative of V is zero, then C_t can be calculated and is equal to $0.5a$. The value for C_t can then be used in Eq.(2) to obtain V_{max}, which is equal to $0.25ac$. The incubation time for V_{max}, $T_{V_{max}}$, can be calculated from the logistic formula and is equal to $1/c$ lnb.

The exponential model is:

$$C_t = a \times e^{ct} \quad (4)$$

The reaction velocity can be calculated by the following differential equation:

$$V = \frac{dC_t}{dt} = c \times C_t \quad (5)$$

In the logistic model, a is the largest limit of C_t, but in the exponential model, a indicates the Fe(II) concentration at day 0 of the incubation, which is equivalent to the amount of ferric iron in the inocula. In both models, parameter c is the reaction velocity constant.

2 Results

2.1 Ferrous iron accumulation in cultures of three paddy soil solutions

There were differences in the effect of substrates on reduced iron accumulation among the three soil solution cultures. Reduced iron accumulation increased quickly in the Jiangxi paddy soil solution culture, when pyruvate, glucose, and citrate were used as the sole carbon source (Fig.1a). In the pyruvate treatment, significant amounts of ferrous iron accumulated from day 13 to day 19 and then gradually moved to equilibrium. The Fe(II) accumulation curve in the citrate treatment was similar to the curve for pyruvate, except that the final Fe(II) accumulation was considerably lower in the citrate treatment. In the glucose treatment, there was a slight increase in Fe(II) during the early part of the incubation period, but Fe(II) increased rapidly from day 27 until the end of the sampling period. A small amount of Fe(II) was detected at the end of the incubation period in the succinate, acetate, or formate treatments, but Fe(II) accumulation in the propionate treatment was negligible, indicating that propionate could not be used as an electron donor by bacteria in the Jiangxi paddy soil.

The number of carbon atoms in these substrates was different; therefore, we calculated the amount of reduced Fe(III) per carbon atom and plotted the results against incubation time (Fig.1b). In the pyruvate treatment, the amount of accumulated Fe(II) per carbon atom increased during the incubation, and at equilibrium, it was considerably higher than in the glucose and citrate treatments. It

Table 1 Chemical properties of soil samples used as inocula

Soil	Sample site		Soil type	Total iron (g/kg)	Free iron (g/kg)	OM (g/kg)	Nitrate (mmol/kg)	Sulfate (mmol/kg)
	Latitude (°)	Longitude (°)						
JX	27.2635	114.7211	Fe-leachi-stagnic anthrosols	19.5	6.5	23.9	59	282
SC	30.3037	103.6872	Fe-accumuli-stagnic anthrosols	34.2	11.7	48.9	2,215	1,407
JL	43.7565	126.4867	Typic-hapli-stagnic anthrosols	34.8	10.1	41.2	4	961

JX, SC, and JL in the column represent the soil extracts from the Jiangxi, Sichuan, and Jilin paddy soils, respectively. OM: organic matter.

needs to be pointed out that although little ferrous iron was detected in the formate treatment, the production of Fe(II) per carbon atom in the formate treatment was the highest among all the treatments, and at the end of the incubation, Fe(II) accumulation per carbon atom was even higher than in the pyruvate treatment.

Ferrous iron started accumulating more rapidly in the Sichuan paddy soil culture after the addition of carbon substrate compared to the Jiangxi paddy soil cultures (Fig.2a). There was significant Fe(II) accumulation within a week in the pyruvate, acetate, and succinate treatments and within 13 d in the glucose and citrate treatments. Fe(II) accumulation in the glucose treatment increased linearly throughout the incubation but Fe(II) accumulation in the citrate treatment reached equilibrium after 27 d.

Calculation of Fe(II) accumulation per carbon atom in each treatment showed that the curves for the pyruvate and acetate treatments were similar (Fig.2b). During the first 15 d of incubation, Fe(II) accumulation per carbon atom was higher in the pyruvate treatment compared to the acetate treatment, but the opposite trend was observed during the rest of the incubation. Ferrous iron accumulation in the formate treatment was significantly lower than in the pyruvate and acetate treatments during the first 27 d of incubation, but then increased rapidly. At the end of the incubation, Fe(II) accumulation was higher in the formate treatment than in any other treatment. When glucose was the sole carbon source, Fe(II) accumulation per carbon atom was initially slow, but then increased rapidly after day 13. At the end of the incubation, Fe(II) accumulation was higher in the glucose treatment than in any of the organic acid treatments with the exception of formate.

In contrast to the Sichuan paddy soil, bacteria in the Jilin paddy soil reacted to the substrates quite slowly (Fig.3a). There was no significant Fe(II) accumulation in any treatment until day 16. From day 16 to day 22, Fe(II) accumulation in the pyruvate treatment dramatically increased and reached equilibrium quickly. When citrate was used as substrate, Fe(II) accumulated slowly and reached equilibrium rapidly after day 30. In the glucose treatment, Fe(II) accumulated slowly during the first 27 d of incubation and then increased linearly until the end of

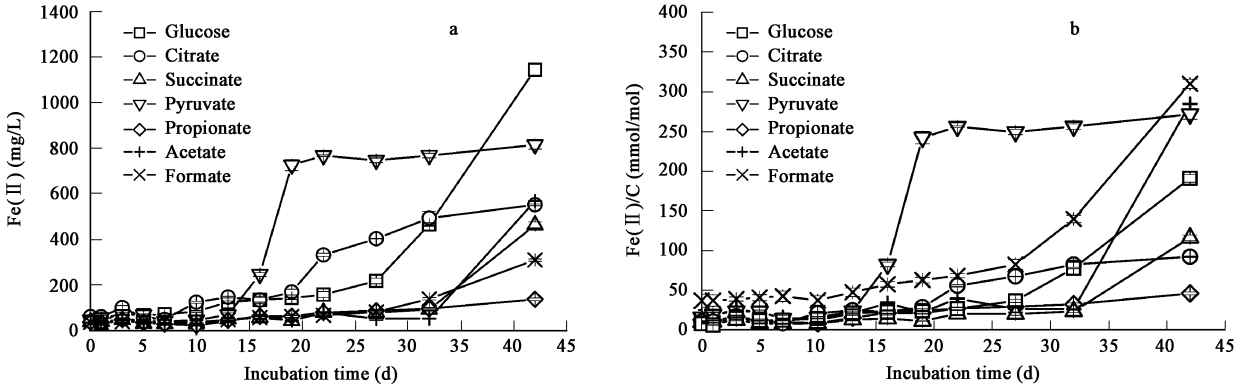

Fig. 1 Fe(II) (a) and Fe(II) accumulation per carbon atom (b) in Jiangxi paddy soil solutions cultured with different substrates.

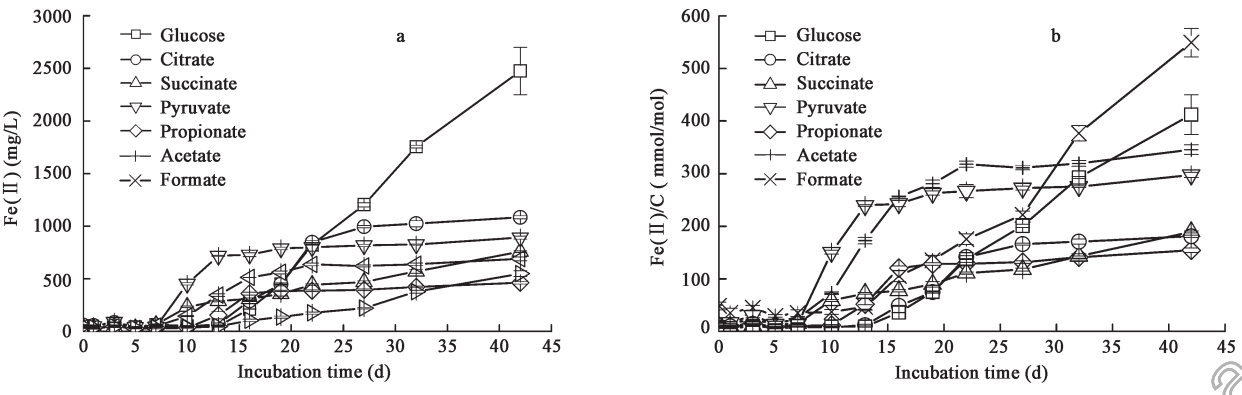

Fig. 2 Fe(II) (a) and Fe (II) accumulation per carbon atom (b) in cultures of Sichuan paddy soil solutions with different substrates.

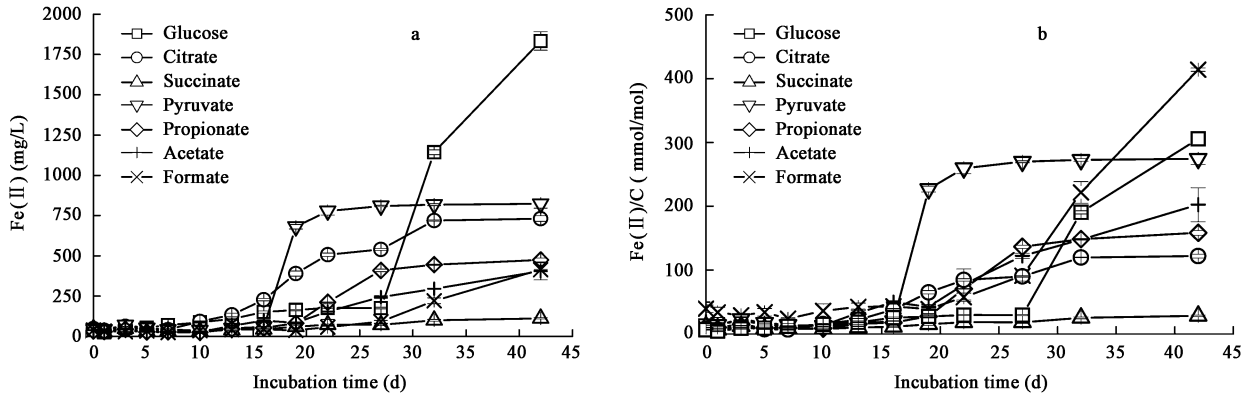

Fig. 3 Fe(II) (a) and Fe(II) accumulation per carbon atom (b) in cultures of Jilin paddy soil solutions with different substrates.

the experiment. The curves for Fe(II) accumulation in the acetate and formate treatments were similar; both exhibited slight increases in Fe(II) on day 27. Fe(II) accumulation in the succinate solution was negligible, indicating that succinate could not be used as an electron donor by the bacteria in the Jilin paddy soil.

Comparisons of Fe(II) accumulation per carbon atom in these treatments indicated that the pyruvate treatment reached equilibrium quickly (Fig.3b). Fe(II) accumulation per carbon atom in the glucose and formate treatments increased during the latter part of the incubation.

2.2 Dynamics

The dynamics of ferrous iron in these treatments were well fitted by logistic and exponential models, but the logistic model provided the most satisfying results (Table 2). Parameter c in the two formulas represented the reaction constant for Fe(III) reduction. The coefficient of variation of the reaction constants in the glucose treatments ranged from 18.9% to 88.5%, suggesting diversity in composition and metabolism among the microbial communities derived from the three soils. In the logistic model, the biological meaning of a is the maximum ferrous iron accumulation, the maximum reaction rate (V_{max}) is equivalent to $0.25a \cdot c$, and the incubation time for V_{max} ($T_{V_{max}}$) is $1/c \ln b$.

Cultures from the Sichuan paddy soil solution showed the greatest capacity to utilize glucose and short organic acids. The large values of a and V_{max}, and the low values of $T_{V_{max}}$ indicated that bacteria reduced ferric iron more rapidly in the Sichan paddy soil compared to the Jilin and Jiangxi paddy soils. The considerably higher reduction activity in the Sichan paddy soil solution can also be attributed to higher variation among the reaction constants in the glucose, citrate, propionate, and acetate treatments.

Compared to the Sichuan paddy soil solution, microbial reduction of ferric iron was lower in the Jiangxi and Jilin paddy soil solutions. In the Jiangxi paddy soil culture, V_{max} in the propionate treatment was only 1.801 mmol Fe(II)/(mol C·d), indicating that the bacteria in the Jiangxi paddy soil did not use propionate as an electron donor.

Table 2 Parameters for the logistic or exponential models of Fe(II) accumulation per carbon atom

Carbon substrate	Inoculum	Model equation	a	b	c			r	SD	V_{max} (mmol/(mol C·d))	$T_{V_{max}}$ (d)
						mean	CV (%)				
Glucose	JX	E	4.252	–	0.090			0.994	5.930	–	–
	SC	L	439.5	158.0	0.182	0.122 B	42.62	0.997	11.02	19.99	27.82
	JL	E	6.027	–	0.094			0.962	25.82	–	–
Citrate	JX	L	104.4	17.7	0.124			0.980	6.434	3.237	23.18
	SC	L	176.9	1,328.0	0.375	0.233 B	55.24	0.994	8.717	16.59	19.18
	JL	L	123.4	47.3	0.200			0.992	6.025	6.171	19.29
Succinate	JX	E	1.398	–	0.104			0.962	8.228	–	–
	SC	L	188.6	12.5	0.126	0.090 B	48.84	0.977	13.66	5.940	20.04
	JL	L	81.18	9.7	0.041			0.936	2.636	0.8322	55.49
Pyruvate	JX	L	259.7	2.1×10^7	1.011			0.992	16.40	65.64	16.70
	SC	L	270.3	904.3	0.690	0.873 A	18.92	0.991	17.59	46.63	9.865
	JL	L	273.1	1.1×10^7	0.918			0.992	17.06	62.67	17.71
Propionate	JX	L	175.7	16.1	0.041			0.958	3.429	1.801	67.81
	SC	L	138.4	8,308.0	0.662	0.351 B	88.46	0.988	10.36	22.90	13.63
	JL	L	158.3	2,681.0	0.350			0.989	9.394	13.85	22.56
Acetate	JX	E	0.3022	–	0.163			0.959	21.52	–	–
	SC	L	324.8	130.3	0.378	0.222 B	61.20	0.996	12.93	30.69	12.88
	JL	L	237.8	33.2	0.126			0.991	9.173	7.491	27.79
Formate	JX	E	18.07	–	0.067			0.981	15.22	–	–
	SC	L	767.9	41.2	0.111	0.087 B	25.60	0.993	20.22	21.31	33.50
	JL	E	12.85	–	0.083			0.983	21.33	–	–

E stands for the exponential model and L stands for the logistic model; "–" indicates no value in the exponential model; A and B indicate the difference in parameter c for each treatment with $P < 0.01$; CV refers to the coefficient of variation for parameter c and is presented as a percentage; SD denotes the standard error estimate in regression models, and r defines the correlation coefficient.

For the Jilin paddy soil culture, Fe(II) accumulation in the succinate treatment was 81.18 mmol/mol C at the end of the incubation period. Its V_{max} was only 0.8322 mmol Fe(III)/(mol C·d), indicating that bacteria in the Jilin paddy soil did not use succinate as an electron donor. Among the treatments, pyruvate was used as a substrate and electron source in ferric iron reduction by cultures derived from all three paddy soils. The mean reaction constant of the pyruvate treatement was significantly larger than in the other treatments and the coefficient of variation (18.92%) was relatively low.

3 Discussion

Previous studies have isolated diverse groups of DFRB with the ability to use a wide spectrum of substrates for Fe oxide reduction, but this knowledge is predominantly based on the studies of pure cultures. The understanding about patterns of substrate use for microbial Fe(III) reduction at the community level is limited. In this study, we used inocula from soil extracts to simulate Fe(III) reduction by the microbial community. It should be noted that our soils were dried before the start of the experiment. Fresh soil extracts may reflect the structure of the soil microbial community more accurately; however, these communities may fluctuate owing to microbial sensitivity to changes in the environment. The microbial populations in the pre-dried soils used in our experiment may represent the most stable components of the microbial community.

In this study, it was found that bacteria from these three paddy soils were uniformly able to use glucose under Fe(III) reducing conditions. The large quantity of ferrous iron accumulation observed at the end of the incubation period in the glucose treatment may indicate that intermediates, not glucose directly, transferred electrons to Fe(III) oxide. Previous reports indicated that only a few DFRB isolates were capable of using glucose directly (Lovley and Coates, 2000). Using isotope-labeled glucose, Chidthaisong *et al*. (1999) found that glucose was mainly degraded into acetate and CO_2 in paddy soil, and 50%–80% of the acetate in the soil was a glucose metabolite. Several bubbles which lifted Fe(III) oxide to the top of the culture solution in the glucose treatment vials were observed in this study. By the end of the incubation, the Fe(III) oxide had been transformed into gray or white granules, not black as we observed in the vials for the other treatments. The gas bubbles may have been a mixture of CO_2 and H_2 produced during glucose fermentation.

There are reports of succinate metabolism by some DFRB, but fewer DFRB have been found to metabolize citrate (Fredrickson *et al*., 2000; King and Garey, 1999). Citrate could be used under Fe(III) reducing conditions, but it had lower capacity to transfer electrons to Fe(III) than glucose. Citrate and succinate are components in the TCA (tricarboxylic acid) cycle, which may be repressed under anaerobic conditions; thus, the use of these substrates in Fe(III) reduction may be blocked.

Pyruvate is the terminal product of glycolysis and has a diverse metabolic pathway. In this experiment, pyruvate also exhibited excellent capacity to transfer electrons to Fe(III) oxide. Propionate and acetate are often observed in anaerobic environments, which contribute greatly to methanogenesis. In particular, there are reports of the direct use of acetate by methanogens as well as DFRB. Propionate is metabolized from acetate under anoxic conditions but with a considerably higher energy barrier compared to acetate (Lueders and Fridrich, 2002). In present study, acetate was more availabile in Fe(III) reduction and produced greater amounts of Fe(II) compared to propionate. Almost no Fe(II) was detected in the propionate treatment of the Jilin paddy soil solution culture, indicating that proprionate was not used for Fe(III) reduction by bacteria in that soil.

Formate is also an important precursor of methanogensis (Frank *et al.*, 2002). There was little Fe(II) accumulation in the formate treatments were found in this study, but Fe(II) accumulation per carbon atom was high, even exceeding the values for pyruvate and acetate. Myers and Myers (1997) isolated a protein containing cytochrome c from the outer membrane of *Shewanella putrefaciens* MR-1, which could be reduced by formate and then transfer electrons to Fe(III) or Mn(IV) oxides. This suggested that formate may play a crucial role for electron transfer in Fe(III) reduction.

Some growth curves showed satisfactory fitting results for the dynamics of Fe(III) reduction. The logistic model provided the most interesting information about Fe(III) reduction and was a powerful analysis tool. In some cases, the exponential model provided reasonable fitting results, especially for reactions in which the accumulation of reduced Fe was low.

Under Fe(III) reducing conditions, there were significant differences in ferrous iron accumulation among the three paddy soils treated with glucose, citrate, succinate, pyruvate, propionate, acetate, or formate. Cultures from the Sichuan paddy soil solutions responded to these substrates quickly and showed the highest reductive activity among the three soil types. There was relatively little evidence of electron transfer to Fe(III) from propionate in the Jiangxi paddy soil culture or succinate in the Jilin paddy soil solution. Differences in substrate use reflected variation in the structure, metabolism, and function of the microbial community among these paddy soils. Pyruvate was readily used by bacteria in all three paddy soil solutions under Fe(III) reducing conditions, indicating that there were some similarities in substrate utilization by bacteria among all soils. The observation that glucose can be used readily for Fe(III) reduction and citrate can be used sometimes demonstrates the important ecological implications of this respiration pathway.

4 Conclusions

Fluctuations in the water table result in the alternating cycles of iron oxidation and reduction in paddy soils. This can have important influences on the adsorption and desorption of ions onto soil colloids as well as other physical and chemical events in the soils. We found large variation in Fe(III) reducing activity among the microbial

communities of different paddy soils. It was pyruvate, not acetate, which primarily contributed to Fe(III) reduction and produced the largest amount of ferrous iron. The observation that glucose and citrate can be metabolized by bacteria and transfer electrons to Fe(III) suggests an even more crucial role of Fe(III) reduction under anoxic environments than was previously believed.

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