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Repetitive sequence based polymerase chain reaction to differentiate close bacteria strains in acidic sites

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Abstract: To study the diversity of bacteria strains newly isolated from several acid mine drainage(AMD) sites in China, repetitive sequence based polymerase chain reaction (rep-PCR), a well established technology for diversity analysis of closely related bacteria strains, was conducted on 30 strains of bacteria *Leptospirillum ferriphilium,* 8 strains of bacteria *Acidithiobacillus ferrooxidans*, as well as the *Acidithiobacillus ferrooxidans* type strain ATCC (American Type Culture Collection) 23270. The results showed that, using ERIC and BOX primer sets, rep-PCR produced highly discriminatory banding patterns. Phylogenetic analysis based on ERIC-PCR banding types was made and the results indicated that rep-PCR could be used as a rapid and highly discriminatory screening technique in studying bacterial diversity, especially in differentiating bacteria within one species in AMD.

Key words: rep-PCR; diversity; acid mine drainage; *Acidithiobacillus ferrooxidans; Leptospirillum ferriphilium*

1 Introduction

Study on microbial community composition in acid mine drainage(AMD) provides important information on community constitution and the relative abundance of each species, which may further indicate the community function and the interaction between the microbial species[1−3]. *Acidithiobacillus ferrooxidans* and *Leptospirillum ferriphilium* are two major populations in the extreme AMD environment. Generally, diversity analysis of microorganism in AMD is based on fabrication of clone library, combined with fingerprinting technology such as RFLP and sequencing of molecular markers like 16SrDNA and *gyr*B gene[4−5]. At species level, these methods have produced excellent data for analysis of community constitution. However, when sub-species level, or even strain level resolution is desired, the use of these methods is limited and genomic scale analysis is often chosen. Based on PCR-mediated amplification of DNA fragments located between specific interspersed repeated sequences in prokaryotic genomes, repetitive sequence based polymerase chain reaction(rep-PCR) fingerprinting is a well established technology in diversity analysis of very closely related species, especially in differentiation of bacteria strains within one species[6−7]. However, although rep-PCR has already been applied extensively in diversity and taxonomic analysis of various bacteria species, the application of this method in analysis of environmental bacteria, especially for bacteria dwelling in the extreme AMD system was rare[8−9]. In this study, rep-PCR using BOXA1R and ERIC primer sets was employed to analyse the diveristy of 38 strains of *Acidithiobacillus ferrooxidans* and *Leptospirillum ferriphilium* newly isolated from several acidic sites in Dexing Copper Mine, China. Reproducibility of the rep-PCR was tested and phlogenetic analysis based on the banding patterns of ERIC-PCR was made. This will provide useful information for researchers interested in microbial ecology and bioleaching as it not only describes the diversity within the species level, which is rare in diversity research, but also targets on the predominant bacteria species in AMD and bioleaching systems.

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2 Experimental

2.1 Bacteria strains and cultivation

Totally, 39 bacteria strains were analyzed in this experiment. The detailed information about the bacteria strains is listed in Table 1. All these bacteria strains were isolated from several acidic sites in Dexing Copper Mine, Jiangxi Province, China. Among them, there were 9 strains of *Acidithiobacillus ferrooxidans*, including 8 strains isolated recently from site Yangtaowu(YTW), and *Acidithiobacillus ferrooxidans* type strain acquired from American Type Culture Collection(ATCC), strain 23270 and 30 strains of *Leptospirillum ferriphilium*. All *Acidithiobacillus ferrooxidans* strains were cultivated using the typical 9K medium ($(NH_4)_2SO_4$ 3.0 g/L, KCl 0.1 g/L, K_2HPO_4 0.5 g/L, $MgSO_4·7H_2O$ 0.5 g/L, $Ca(NO_3)_2$ 0.01 g/)) with $FeSO_4·7H_2O$ (44.22 g/L) and pH adjusted to 3.0 under the temperature of 30 ℃, while *Leptospirillum ferriphilium* strains were cultivated using 9K medium with $FeSO_4·7H_2O$ (44.7 g/L) and pH adjusted to 1.6 under 40 ℃.

Table 1 Bacteria strain and corresponding site isolated

Strain	Species	Site
1	*Leptospirillum ferriphilium*	Dawutou
2	*Leptospirillum ferriphilium*	Dawutou
3	*Leptospirillum ferriphilium*	Dawutou
4	*Leptospirillum ferriphilium*	Dawutou
5	*Leptospirillum ferriphilium*	Dawutou
6	*Leptospirillum ferriphilium*	Dawutou
7	*Leptospirillum ferriphilium*	Shuilongshan
8	*Leptospirillum ferriphilium*	Shuilongshan
9	*Leptospirillum ferriphilium*	Shuilongshan
10	*Leptospirillum ferriphilium*	Shuilongshan
11	*Leptospirillum ferriphilium*	Shuilongshan
12	*Leptospirillum ferriphilium*	Shuilongshan
13	*Leptospirillum ferriphilium*	Yangtaowu
14	*Leptospirillum ferriphilium*	Yangtaowu
15	*Leptospirillum ferriphilium*	Yangtaowu
16	*Leptospirillum ferriphilium*	Yangtaowu
17	*Leptospirillum ferriphilium*	Yangtaowu
18	*Leptospirillum ferriphilium*	Yangtaowu
19	*Leptospirillum ferriphilium*	Yangtaowu
20	*Leptospirillum ferriphilium*	Yangtaowu
21	*Leptospirillum ferriphilium*	Yangtaowu
22	*Leptospirillum ferriphilium*	Dawutou
23	*Leptospirillum ferriphilium*	Dawutou
24	*Leptospirillum ferriphilium*	Dawutou
25	*Leptospirillum ferriphilium*	Shuilongshan
26	*Leptospirillum ferriphilium*	Shuilongshan
27	*Leptospirillum ferriphilium*	Yangtaowu
28	*Leptospirillum ferriphilium*	Yangtaowu
29	*Leptospirillum ferriphilium*	Yangtaowu
30	*Leptospirillum ferriphilium*	Shuilongshan
31	*Acidithiobacillus ferrooxidans*	Yangtaowu
32	*Acidithiobacillus ferrooxidans*	Yangtaowu
33	*Acidithiobacillus ferrooxidans*	Yangtaowu
34	*Acidithiobacillus ferrooxidans*	Yangtaowu
35	*Acidithiobacillus ferrooxidans*	Yangtaowu
36	*Acidithiobacillus ferrooxidans*	Yangtaowu
37	*Acidithiobacillus ferrooxidans*	Yangtaowu
38	*Acidithiobacillus ferrooxidans*	Yangtaowu
39	*Acidithiobacillus ferrooxidans* ATCC 23270	

2.2 rep-PCR and gel electrophoresis

Genomic DNAs of all bacteria strains were extracted using the Bacteria Genomic DNA Extraction Kit (Tiangen Corporation Ltd. Beijing, China) at 48 h after inoculation. BOX-PCR (based on primers targeting the highly conserved repetitive DNA sequences of the BOXA subunit of the BOX element) and ERIC-PCR (based on primers targeting the highly conserved enterobacterial repetitive intergenic consensus) were conducted to obtain the genomic fingerprinting of the bacteria described above. The primers used were: for ERIC-PCR, ERIC-1R: 5'-ATGTAAGCTCCTGGGGA-TTCAC-3', ERIC-2: 5'-AAGTAAGTGACTGGGGTG-AGCG-3', and for BOX-PCR, BOXA1R: 5'CTACGGC-AAGGCGACGCTGACG-3'. Polymerase chain reaction (PCR) was done in 50 μL reaction solution containing 50 pmol each of the two primers for ERIC-PCR and 100 pmol BOX primer for BOX-PCR, 5.0 μL of 10×PCR buffer, 400 μmol/L dNTPs, 5.0 mmol/L $MgCl_2$, 2 U of *Taq* DNA polymerase (Fermentas Corporation, Ltd. USA), and 60 ng of template DNA. PCR reaction was performed in a thermocycler (Applied Biosystems Corporation, Ltd., USA). The reaction procedure was: an initial denaturation at 95 ℃ for 5 min, followed by 40 cycles of denaturation at 90 ℃ for 30 s, annealing at 50 ℃ (for BOX-PCR) or 42 ℃ (for ERIC- PCR) for 30 s, extension at 72 ℃ for 8 min and a final extension at 72 ℃ for 10 min. PCR products were then examined through horizontal electrophoresis in 2% agarose gel containing ethidium bromide (0.5 μg/mL) at 60 V for 6 h in 1× TAE buffer. Gel images were captured using the Biosense System (Fushan Corporation Ltd, Shanghai, China) and then stored as tif file for further analysis.

2.3 Phylogenetic analysis and statistical method

Band counting was accomplished using software Quantity One (Bio-Rad Corporation, Ltd., USA). The relative distance of each band was recorded. Based on

the banding patterns recognized on gel images, a binary (0-1) data sheet was set up, the similarity matrix was calculated and the cluster analysis was made as described by Rademaker et al[8], using the software NTSYS 2.0.

3 Results

3.1 Comparison of rep-PCR results using BOXA1R primer set and ERIC primer set

The result of electrophoresis is shown in Fig.1. Generally, rep-PCR using both BOXA1R and ERIC primer sets produced similar, but not identical banding types for bacteria strains in both bacteria species. However, differences existed in different bacteria strains within one species using the same primer set, indicating the high resolution of rep-PCR in analysis of very closely related bacteria strains within these two species. The size of amplification products in BOX-PCR ranged from around 100 bp to nearly 8 kb, while band sizes produced through ERIC-PCR varied from less than 100 bp to larger than 8 kb (8 kb is the largest fragment of the DNA marker used in this study).

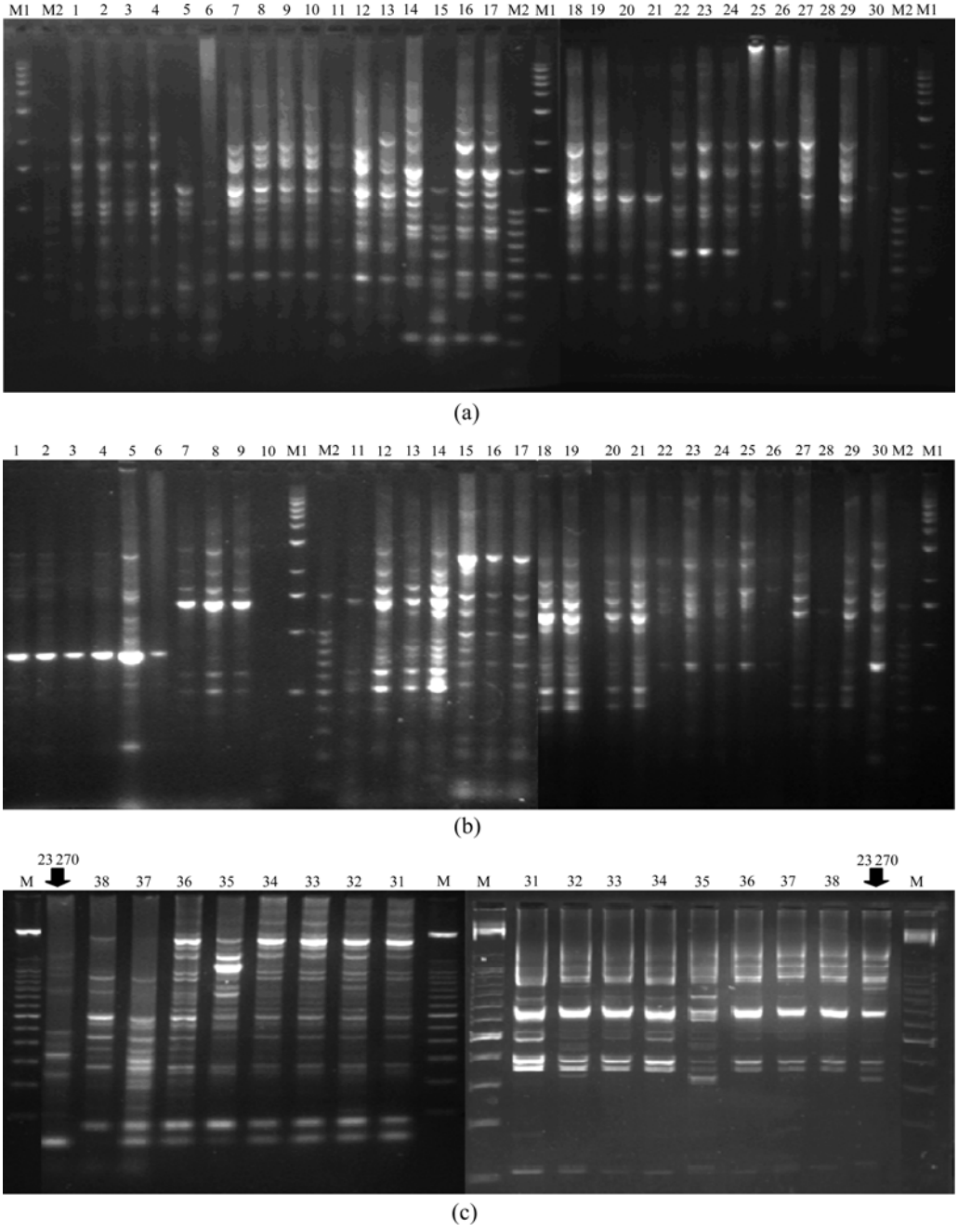

Fig.1 rep-PCR banding types using BOXA1R primer set and ERIC primer set: (a) rep-PCR banding types using BOXA1R primer set for 30 strains of *Leptospirillum ferriphilium*; (b) rep-PCR banding types using ERIC primer set for 30 strains of *Leptospirillum ferriphilium*; (c) rep-PCR banding types using ERIC (left) and BOXA1R (right) primer sets for 9 strains of *Acidithiobacillus ferrooxidans* (M2 and M1 are 100 bp and 1 kb DNA markers, respectively; M is 3 kb DNA marker)

Totally, BOX-PCR produced 419 bands (10.74 bands per bacteria strain), while 494 bands (12.67 bands per bacteria strain) were produced in EIRC-PCR. However, in 30 strains of *Leptospirillum ferriphilium*, the numbers of bands produced by BOX-PCR and ERIC-PCR were 313 and 341 bands, respectively, while in 9 strains of *Acidithiobacillus ferrooxidans*, ERIC-PCR produced 153 bands, compared with 106 bands produced by BOX-PCR. Thus, ERIC-PCR set may produce more complicated banding patterns, indicating ERIC-PCR may have higher discriminatory power in this study. Therefore, fingerprinting produced by ERIC-PCR primer set was chosen for cluster analysis.

3.2 Phylogenetic analysis based on ERIC-PCR banding types

Cluster analysis of 39 bacteria strains was made based on the banding types of ERIC-PCR. Band counting was done also using the Quantity One software. The relative distance of every band on gel was recorded. All relative distance data were then collected and a 0-1 binary data sheet (not shown here) was set up according to the banding distribution, where "1" represents the occurrence of a band at the specific location while "0" indicates that no band was detected at the corresponding location. Similarity matrix was calculated based on the data sheet and UPGMA algorithm was employed for cluster analysis, using software NTSYS2.0. The result is shown in Fig.2. From Fig.2, 39 bacteria strains were clustered into 2 major groups: Group 1 and Group 2. All the 9 strains of *Acidithiobacillus ferrooxidans* were clustered together in Group 1, while all the 30 strains of *Leptospirillum ferriphilium* were clustered in Group 2, indicating that rep-PCR using ERIC primer sets can well differentiate these two bacteria species. At the sub-species level, most, but not all bacteria strains fell into different clusters, demonstrating the high resolution of ERIC-PCR. However, strain level resolution was not obtained for both bacteria species.

3.3 Evaluation of reproducibility

Although the reproducibility of rep-PCR is confirmed in analysis of various microorganism [9−12], experiment was done in this study to test the reproducibility of both BOX-PCR and ERIC-PCR targeting bacteria species dwelling in AMD. The bacteria strain was randomly chosen from both *Acidithiobacillus ferrooxidans* and *Leptospirillum ferriphilium*. All runs were performed using the same thermal cycler. The results indicated that repeated BOX-PCR or ERIC-PCR produced banding patterns of similarity within the range of 93.2%−97.1% (data not shown). Moreover, the dissimilarity was mainly due to the differences of the signal intensity of a specific band, rather than the missing or occurrence of a band. These results indicated that the reproducibility of both BOX-PCR and ERIC-PCR was good in the microbial species *Acidithiobacillus ferrooxidans* and *Leptospirillum ferriphilium*. The variation observed did not significantly affect the result of cluster analysis.

4 Discussion

The rep-PCR technology, which targets on several repetitive sequences in bacteria, has a wide range of application in diversity analysis of bacterium. Compared with other genomic fingerprinting techniques, such as RFLP and NAPD, the comprehensive simplicity, low

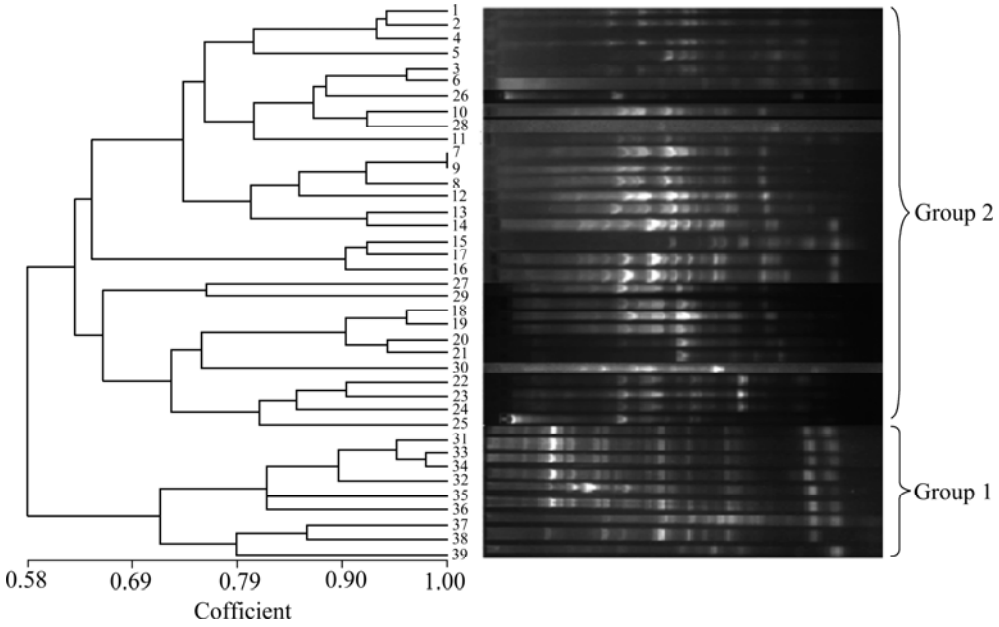

Fig.2 Phylogenetic analysis based on ERIC-PCR banding patterns

cost, high resolution and reliability of rep-PCR made it advantageous, especially when the targeting bacteria strains were closely related. Using 178 strains of genus *Xanthomonas* as a model system, RADEMAKER et al [13] compared the result of rep-PCR with DNA-DNA homology analysis. High correlation was observed between rep-PCR-derived relationships and DNA-DNA homology values, indicating genomic fingerprinting using rep-PCR technology is a rapid, highly discriminatory and reliable screening technique to determine the taxonomic diversity and phylogenetic structure of bacterial populations. CLELAND et al[14] applied rep-PCR in genotyping and identification of 79 strains representing 27 genera of Archaea. Strain-level resolution was observed and the reproducibility between rep-PCR reactions was confirmed. However, the use of rep-PCR has been constrained primarily to bacterial taxa that are of importance to human, animal, or plant health. Relatively little work has been done to determine the efficacy of using rep-PCR to identify the broader diversity of environmental microbes, especially in analysis of bacteria species inhabiting in the extreme AMD system.

In this study, rep-PCR was conducted to analyze 38 bacteria strains recently isolated from a typical AMD system in Dexing Copper Mine, China, including 30 strains of *Leptospirillum ferriphilium* and 8 strains of *Acidithiobacillus ferrooxidans*, as well as the *Acidithiobacillus ferrooxidans* type strain ATCC 23270. Both BOX-PCR and ERIC-PCR produced good banding patterns of excellent discriminatory power. But the banding patterns produced by ERIC-PCR were more complicated than those by BOX-PCR. Therefore, ERIC-PCR banding patterns were used for cluster analysis. The results again proved the excellent discriminatory power of ERIC-PCR. The 39 bacteria strains involved in this study were clustered into two major groups, all 30 strains of *Leptospirillum ferriphilium* clustered together and all 9 strains of *Acidithiobacillus ferrooxidans* clustered together, demostrating the reliability of using rep-PCR in phylogenetic analysis of these two bacteria species, which were major populations in the AMD system. Besides, high resolution was obtained at the sub-species level in ERIC-PCR. All 30 strains of *Leptospirillum ferriphilium* fell into 19 clusters and 9 strains of *Acidithiobacillus ferrooxidans* fell into 6 clusters. However, not as expected and reported by some previous studies, strain-level resolution was not obtained by ERIC-PCR for both species involved in this study. This may be explained by the fact that the bacteria strains cannot be differentiated which were isolated from the same site, such as strains 1 and 2, strains 7 and 9, strains 15 and 17 and strains 20 and 21, resulting in the extremely high homology between the strains; while in previous studies[15−18], the bacteria strains were generally isolated from different sites with various distances and geochemical characteristics.

Interestingly, from the cluster analysis, some strains isolated from the same site clustered together, such as strains 1, 2, 4, 5 from site DWT; strains 7, 8, 9, 12 from site SLS; strains 15, 16, 17 and strains 18, 19, 20, 21 from site YTW; while other strains, also isolated from one site, were scattered into different clusters. As rep-PCR targets on the entire bacteria genome, the cluster result also revealed the genomic variation of bacteria at the sub-species, or strain level, even when these microbes were isolated from the same site. Further study is needed to evaluate the potential application of rep-PCR in elucidation of the relationship of microbial population and site geochemistry.

5 Conclusions

As a highly discriminatory, accurate, fast and low-cost technique, rep-PCR provides a powerful and convenient tool to analyse bacteria diversity. BOXA1R and ERIC primer sets were sued to perform rep-PCR on 39 AMD bacteria strains belonging to species *Leptospirillum ferriphilium* and *Acidithiobacillus ferrooxidans*. The results indicate that both BOX-PCR and ERIC-PCR have excellent discriminatory power at the species level. Cluster analysis based on ERIC-PCR banding patterns found that all 39 bacteria strains clustered into two major groups. All the 30 strains of *Leptospirillum ferriphilium* clustered together and all 9 strains of *Acidithiobacillus ferrooxidans* grouped together. However, despite the relatively high resolution, strain level discriminatory power was not obtained. The very close geographic distance between the AMD sites where the bacteria strains were isolated, and similar geochemical characteristics may be responsible for the extremely high homology between these bacteria strains.

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