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Direct RAPD Evaluation of Bacteria without Conventional DNA Extraction

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ABSTRACT

The present work reports successful DNA amplification of Pantoea agglomerans *and* Bacillus pumilus *through Random Amplified Polymorphic DNA (RAPD). For this, template DNA was obtained without conventional DNA extraction. The procedure was as follows: cultures grown for 20 hours in 5 mL LB medium were centrifuged and the resulting preparation was suspended in TE buffer. After boiling, the cell suspension was diluted and 2.0 µl were used in reactions of 15 µl. The results showed no significant differences among the RAPD profile of the PCR reactions derived from the boiling and phenol extraction methods, suggesting the utilization of this method for genetic population analysis.*

Key words: Bacteria, boiling method, DNA extraction, RAPD

INTRODUCTION

RAPD (Random Amplified Polymorphic DNA) technique is a powerful tool for genetic studies and has been simultaneously described by Williams et al. (1990) and Welsh and McClelland (1990). However, its use for genetic population analyses may be limited by the laborious procedures involved in the extraction of genomic DNA from large sets of samples. More simplified techniques were developed to overcome this problem, as those associating formamide to heating (Panaccio et al., 1993) or the use of specific buffers (Steiner et al., 1995; Hilton et al., 1997).

Recently, procedures such as boiling have been used to promote cell lysis and detect pathogens in plant tissues (Zhang and Goodwin, 1997; Sulzinski et al., 1997), or also to inactivate compounds such as proteinase K, which may inhibit the *Taq* DNA polymerase (Goldenberger et al., 1995). However, these methods are still time consuming.

PCR reactions using DNA obtained by cell boiling is a routine in many laboratories worldwide, however, in RAPD analysis this strategy has not been used. Analyses reported by Stephan et al. (1994) mention a RAPD reaction with DNA of *Bacillus licheniformis* extracted by cell boiling, but little information concerning the efficiency and sensibility of this technique was given. Here we investigate these aspects through the density of cells in each reaction.

MATERIALS AND METHODS

Bacterial isolates

The endophytic bacteria *Pantoea agglomerans* (strain T045) and *Bacillus pumilus* (strain LD31)

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originally isolated from citrus hybrid (*Citrus paradisi* Macf. X *C. reticulata* Blanco) and *C. sinensis* Osbeck, respectively (Araújo et al., 2001) were used in the present analysis.

Determination of bacterial density

Bacteria were grown in 5.0 mL of LB medium (0.5% yeast extract, 1% tryptone, 1% NaCl) and appropriated dilutions were plated on LBA medium (LB plus 1.5% agar) for determination of the number of colony forming units (CFU). Colony counts were made after 24 hours of incubation at 28ºC. At least four repetitions were performed.

Preparation of DNA samples for RAPD

Two methods of DNA extraction were used: phenol extraction method (PM) and boiling cells (BC). For the BC method, bacteria were grown for 20h, until log phase, and were centrifuged (4000 g; 2 min), washed in 0.85% NaCl solution and centrifuged again. The pellet was ressuspended in 1.0 mL of TE buffer (EDTA 1.0 mM; Tris-HCl 10 mM, pH=8.0) and boiled for 15 min. Serial dilutions with Tris-HCl (10 mM, pH=8.0) were made and 2.0 μL of the appropriate dilutions used in RAPD reactions.

For PM, total DNA was extracted from bacterial cultures in LB broth. After 24h, 50 mL of culture was removed and centrifuged at 3000 g for 5 min, after which the cells were washed in 0.85% NaCl solution, re-centrifuged and resuspended in 2 mL of TEN buffer (100 mM EDTA; 150 mM NaCl; 100 mM Tris-HCl, pH=8.0) containing 4 $mg.mL^{-1}$ lysozyme. The suspension was incubated at 37°C for 45 min and 0.5 mL of 8.5% SDS was added, followed by incubation at 75°C for 30 min before the addition of 1.5 mL of potassium acetate (5 M, pH=5.2) and incubation for 20 min at 4°C. The DNA was extracted with phenol – chloroform - isoamylalcohol, precipitated with isopropanol (Sambrook et al., 1989), washed with 70% ethanol, briefly dried and re-suspended in 200 μL of TE buffer.

RAPD analysis

RAPD analysis was carried out in 15 μL final volume, containing 2.5 ng of DNA template or 2.0 μl of diluted boiled bacterial culture, 0.4 mM primer, 200 μM each of dCTP, dGTP, dATP and dTTP (Pharmacia), 5 mM $MgCl_2$ and 1.5 U of *Taq* DNA polymerase (Gibco - Life Technologies) in 20 mM Tris–HCl, pH=8.4 containing 50 mM KCl. The thermal cycling profile was as follows: 4 min initial denaturation at 92°C, 40 cycles of 1 min at 92°C, 2 min at 37°C, 3 min at 72°C, followed by a final extension at 72°C for 3 min. PCR products were analysed in 1.4% agarose gels stained with ethidium bromide. Negative controls contained water instead of DNA.

The primers used were OPE-14 (5'-TCG GGC TGA G-3'), OPE-19 (5'-ACG GCG TAT G-3'), OPE-20 (5'-AAC GGT GAC C-3'), OPX-09 (5'-GGT CTG GTT G-3'), OPX-16 (5'-CTC TGT TCG G-3'), OPE-12 (5'-TTA TCG CCC C-3') and OPX-18 (5'-TGG CAA GGC A-3'), supplied by Operon Technologies (Alameda, CA, EUA).

RESULTS AND DISCUSSION

In the present analysis, RAPD patterns obtained from bacteria had no significant differences between BC and PM methods (Figs. 1 and 2). Besides, the RAPD observed fragments ranged from 0.4 to 2.1 kb, suggesting that long DNA template was kept in the cell suspension, since degraded DNAs usually yielded low molecular weight PCR products (Steiner et al., 1995). These results showed that BC might be helpful in studies using a high number of samples, since it was less expensive than any other methodology currently used.

However, the reduction of bacterial cell number in reaction changed the amplification pattern, increasing the unspecific and no repeatable pattern (Figs 1 and 2). At low number of CFU in the RAPD reaction, a loss of reliable bands was observed, probably due to a minimal concentration of DNA template (Figs. 1 and 2). Similar results were previously observed (Welsh and McClelland, 1990).

The BC method was more sensitive for RAPD analysis of *P. agglomerans,* a Gram-negative bacterium, than in *B. pumilus,* a Gram-positive bacterium. Reproducible RAPD patterns were obtained with 10^5 – 10^3 CFU of *P. agglomerans* cells. At the same cell concentration, the RAPD patterns of *B. pumilus*, a Gram-positive bacterium, were not reproduced. The difference in sensitivity observed could be explained by cell wall differences between these bacteria.

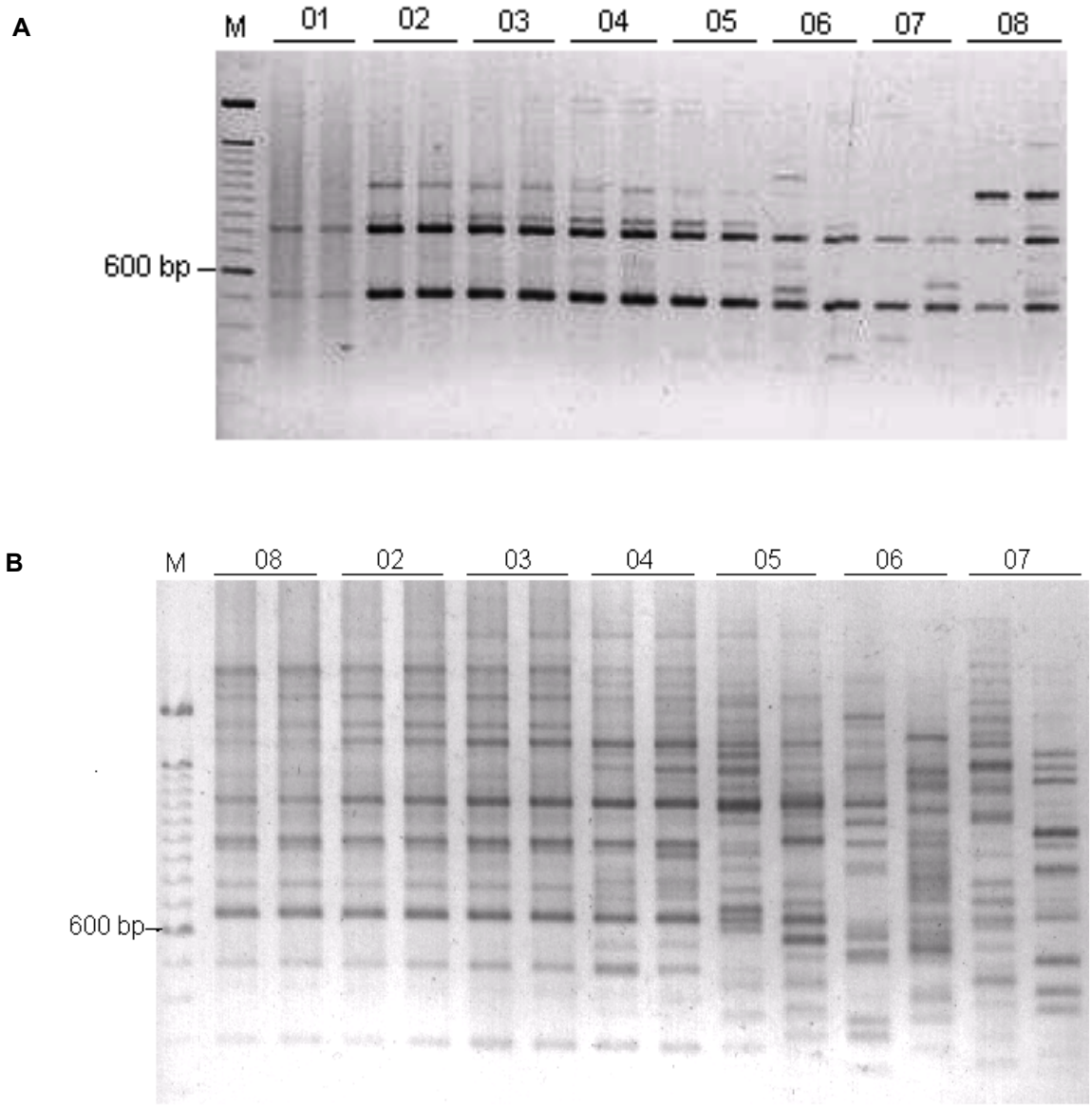

Figure 1 - Variation in *P. agglomerans* RAPD profile using primers (A) OPX-09 (B) OPE-19. Number of cells used per PCR mix: (1) 10^6 CFU; (2) 10^5 CFU; (3) 10^4 CFU; (4) 10^3 CFU; (5) 10^2 CFU; (6) 10^1 CFU; (7) 1 CFU; (8) DNA extracted by PM method; (M) 100 bp DNA ladder (Gibco – Life Technologies).

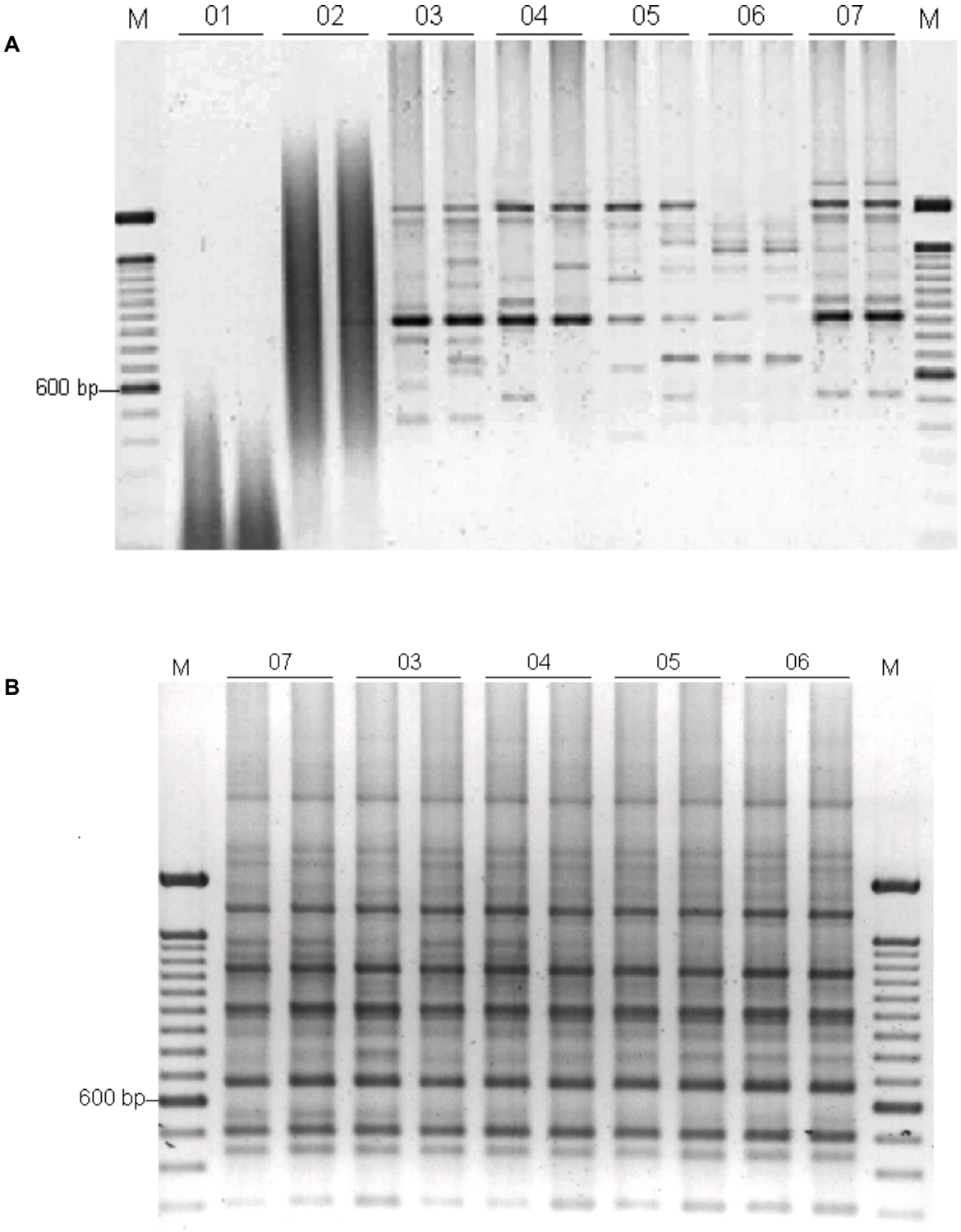

Figure 2 - Variation in *B. pumilus* RAPD profile using primers (A) OPX-09 and OPE-19. Number of cells used per PCR mix: (1) 10^8 CFU; (2) 10^7 CFU; (3) 10^6 CFU; (4) 10^5 CFU; (5) 10^4 CFU; (6) 10^3 CFU; (7) DNA extracted by PM method; (M) 100 bp DNA ladder (Gibco – Life Technologies).

Zhang and Goodwin (1997) reported that the PCR technique might detect 1.25 x 10^3 CFU.ml^{-1} of *Xanthomonas fragariae* suspension, using a method similar to the BC method plus an auxiliary buffer to break the cells. At high cell density, DNA amplification in the RAPD reaction was inhibited, but successive dilutions of boiled cells avoided this inhibition, suggesting that a high DNA concentration or inhibitory compounds could be responsible for this result. The most reliable cell concentration ranged from 10^3 to 10^5 and from 10^5 to 10^6 CFU per PCR mix for *P. agglomerans* and *B. pumilus,* respectively (Figs 1 and 2). More studies should be performed for a better understanding of the details related to the RAPD technique. Here, we determined the number of cells needed for a reliable RAPD reaction.

Other simplified methods to obtain DNA template for PCR have been described. However, the boiling method seems to be very efficient, simple and may be used in the RAPD technique. The low number of steps required in this procedure was one of the positive aspects, because a higher number of samples could be processed per day. Another advantage was that it was not necessary to change tubes during incubation. Besides, the BC method did not produce hazardous wastes (table 1), it was much less expensive and special drugs or equipments were not necessary. Therefore, this study suggest that the BC method was the most convenient method to obtain DNA for genetic population analyses through RAPD markers, which did not need purified DNA. Also, this method could be used with bacterial colonies grown on solid media after bacterial isolation (data not shown).

Table 1 - Comparison of DNA extraction methods for use with RAPD and PCR amplification.

Characteristics	BC[†]	Rose[‡]	PM	Hilton[¶]
Number of samples /day	>3200	1920	52	3000
Number of steps* for extraction	1	2	9	3
Hazardous waste per day (ml) [£]	0	0	300	-
Number of reactions	>5000	13600	5000	5000

*An extraction step is defined as any manipulation (e.g. different incubation), activity requiring the transfer of tube contents or the addition of reagents after the initial extraction solution is added to cells.
[£] Based on optimized number
[†] This work
[‡] Steiner et al. (1995)
[¶] Hilton et al. (1997)

CONCLUSIONS

The BC could be a reliable method to obtain template DNA to use in RAPD analysis. It did not produce hazardous waste and was easier than others methods, which have currently been used in genetic population analysis. However, for routine application, it is recommended to use a range of tenfold dilution in duplicate, establishing the optimal cell number in PCR reaction. This optimisation adds robustness and reliability to the reactions, mainly in analysis of new species. This approach may greatly facilitate epidemiological, genetic and ecological studies of bacteria.

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RESUMO

O presente trabalho mostra a amplificação de DNA das bactérias *Pantoea agglomerans* e *Bacillus pumilus* por meio da técnica de RAPD (Amplificação ao acaso de DNA polimórfico). Para esta análise, o DNA molde foi obtido sem a

utilização de técnicas de extração convencional, ou seja, sem a purificação do DNA. Bactérias foram cultivadas por 20 horas em 5 mL de meio LB, centrifugado e ressuspendido em tampão TE. A suspensão resultante foi fervida por 5 min., diluída e 2,0 μL foram usados em reações de 15 μL. Os resultados mostraram que os padrões observados com o DNA obtido pela fervura das células não apresentou diferenças significativas daquele obtido com DNA extraído e purificado com fenol, sugerindo a possibilidade da utilização deste método para o estudo da variabilidade genética de populações microbianas.

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