

Full Length Research Paper

Molecular characterization of bacteria isolated from the Kingdom of Saudi Arabia and their uses against pathogenic fungi causing dermatological diseases

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Random amplified polymorphic DNA (RAPD) assay and antimicrobial activities were used for the detection of genetic variations of antimicrobial isolates from the Kingdom Saudi of Arabia. Soil samples were collected from El-Madina El-Monawara, El-Taif, El-Jouf and El-Dammam for bacterial isolation. Bacterial isolates were evaluated for their antimicrobial activities against pathogenic fungi which cause dermatological diseases; *Acremonium* sp., *Microsporium gypseum*, *Microsporium gallinae*, *Exserohilum* sp., *Trichphyton* sp. and *Aspergillus niger*. Results indicate that all the bacterial isolates showed different levels of antagonistic activities against the pathogenic fungi. *Esherichia coli* M2, *Bacillus megaterium* M3 and *Bacillus subtilis* M10 showed strong effect against all the tested pathogenic fungi. *B. subtilis* J1 isolate showed strong effect against the two pathogenic fungi *M. gallinae* and *Exserohilum* sp. Nine strains were tested for their antibiotic response. The results show that different antibiotic response was found. RAPD fingerprinting technique was applied to study the genetic diversity among the tested strains. Results indicate that there was a significant genomic diversity among the *B. subtilis* strains and this diversity was highly correlated with habitats and the antagonistic effect of the studied strains.

Key words: Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), bacterial isolates, pathogenic fungi, Kingdom of Saudi Arabia.

INTRODUCTION

The dermatological pathogenic microbes could penetrate the skin through the cracks ulcers causing skin infections. Their treatment depends on different antibiotics, which have several side effects. Bacterial species producing antibiotics have been used as bio-control agents against pathogenic fungi (Yilmaz et al., 2005; Gebreel et al., 2008). Certain bacterial and fungal isolates were isolated from soil samples collected from El-Madina El-Monawara, and showed significant antagonistic effect against

pathogenic microbes causing dermatological diseases (Sheikh, 2010).

Molecular techniques based on genomic DNA have extensively been used for analysis of genetic diversity. When the reproducibility can be stably rendered with replications by the same primers, random amplified polymorphic DNAs (RAPDs) technique is very useful since it is fast, cheap and an easy method in genetic mapping and characterization. One prominent advantage of the RAPD is that it can easily be applied and employed on antagonistic gram (-) and gram (+) bacteria (Cirvilleri et al., 2005; Baysal et al., 2008).

This study is an attempt to investigate the antimicrobial activity of certain microorganisms isolated from the soil

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collected from different areas in the Kingdom of Saudi Arabia (El-Madina El-Monawara, El-Taif, El-Jouf and El-Dammam) against some pathogenic microorganisms which cause wounds inflammation and ulcers such as *Acremonium* sp., *Microsporium gypseum*, *Microsporium gallinae*, *Exserohilum* sp., *Trichphyton* sp. and *Aspergillus niger*. Also, RAPD method was used to investigate the genetic heterogeneity of *Bacillus* and *Escherichia coli* strains isolated from different habitats in relation to their antimicrobial activity against pathogenic fungi causing dermatological diseases.

MATERIALS AND METHODS

Microbial strains, media and culture conditions

Bacterial isolates from soil samples were collected from different localities (El-Madina El-Monawara, El-Taif, El-Jouf and El-Dammam, Kingdom of Saudi Arabia). The pathogenic fungi isolates were obtained from King Fahd-hospital, Jeddah city. The blood agar culture medium recommended by Madigan and Martinko (2005) was used for cultivation of pathogenic bacteria isolates. Sabouraud dextrose agar medium was used for fungal growth (Georg et al., 1954). The bacterial isolates were identified according to Bergey's manual of systematic bacteriology (Krieg and Holt, 1984; Sneath et al., 1986).

Antagonism test

The antagonistic effects of the bacterial species were tested according to the method of Umechuruba and Nwachukwa (1997).

Testing of antibiotic response

The antimicrobial resistance patterns of bacterial strains were routinely tested by the single-disk diffusion method using Muller-Hinton agar against the following antibiotics: Ampicillin (Amp, 25 µg/ml), streptomycin (Sm, 10 µg/ml), tetracycline (Tc, 30 µg/ml) and norfloxacin (Nor, 10 µg/ml), gentamycin (Gm, 10 µg). Zone sizes were interpreted by using standard recommendations.

Isolation of total DNA from isolated strains

Total DNA was isolated according to Sambrook et al. (1989). The quantity and purity of the obtained DNA were determined according to the ultraviolet (UV)-absorbance at 260 and 280 nm using spectrophotometer (Shimadzu UV-VIS model UV-240).

RAPD analysis of new bacterial isolates

Polymerase chain reaction (PCR)-GOLD Master-Mix Beads (BIORON, Germany, Cat. No. 10020-96) were used for PCR technique. Each bead contains all of the necessary reagents, except primer and DNA template, for performing 25 µl PCR amplification reactions. Two different primers were used in the present study. The first primer (No.1) sequence was 5'-GTC CAC ACG G-3'. The second primer (No.2) sequence was 5'-GGT CCC TGA C-3'. All primers were supplied by Operon Technologies Company, Netherlands. To each ready-to-go PCR bead, 12 ng of the used random primer and 40 ng of the purified DNA sample were

added. The total volume of the amplification reaction was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows: denaturation at 95°C for 5 min. 35 cycles each consists of the following steps: denaturation at 95°C for 1 min; primer annealing at 32°C for 2 min according to guanine-cytosine (GC) ratio of each primer and incubation at 72°C for 2 min, for DNA polymerization. At the end, the PCR was held at 4°C till analysis. The amplified DNA products from RAPD analysis were electrophoresed in 1% agarose gel and 1 × TBE buffer at consistent 100 volt for about 2 h. The different band sizes were determined against 100 bp ladder and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using both Polaroid Instant Camera and UV Transilluminator.

RESULTS AND DISCUSSION

Antagonism of isolated strains against dermatological fungi

Nine bacterial strains were isolated from the soil samples collected from different localities from El-Madina Al-Monwara (M2, M3 and M10), El-Taif (T2 and T3), El-Jouf (J1 and J3) and El-Dammam (D2 and D4), Kingdom of Saudi Arabia.

The bacterial strains were screened for their antifungal activity against some pathogenic microorganisms that cause wounds inflammation and ulcers, *Acremonium* sp., *M. gypseum*, *M. gallinae*, *Exserohilum* sp., *Trichphyton* sp. and *A. niger*. Experimental results show that all bacterial isolates had different levels of antagonistic activities against the pathogenic fungi (Table 1). *E. coli* M2, *Bacillus megaterium* M3 and *Bacillus subtilis* M10 showed the strongest effects against all tested pathogenic fungi. *E. coli* M2 showed effective biocontrol potential against all the tested fungi, that is, *Trichphyton* sp., *M. gallinae*, *M. gypseum*, *Exserohilum* sp., *Acremonium* sp., *A. niger*, with the inhibition zones 45, 72, 85, 85, 75 and 15 mm, respectively. For *B. megaterium* M3, the inhibition zones were 70, 69, 60, 75, 85 and 25 mm, respectively. On the other hand, inhibition zones were 65, 85, 67, 65, 60 and 24 mm, respectively, with *B. subtilis* M10. *B. subtilis* J1 and J3 produced inhibition zones of 60, 85, 40, 75, 12 and 20 mm, respectively and 50, 22, 40, 35, 70 and 20 mm. *B. subtilis* J1 showed strong effect against two pathogenic fungi, that is, *M. gallinae* and *Exserohilum* sp. *B. subtilis* D2 and *B. subtilis* D4, produced moderate inhibition zones, whereas, *B. subtilis* T2 and *B. subtilis* T3 could not produce clear antimicrobial activity against the tested pathogenic fungus.

As appeared from the obtained data, *Bacillus* was the dominant genus; these results were supported by several studies which indicated the efficient antimicrobial activity of soil bacteria especially the bacilli. In this connection, Aunpad and Na-Bangchang (2007) concluded that *Bacillus pumilus* isolated from the soil of Thailand produced new antimicrobial peptide with broad spectrum antibacterial activity including methicillin-resistant

Table 1. The antagonistic effects of bacterial suspensions isolated from soil against pathogenic fungi expressed by inhibition zones (mm).

Bacterial isolate	Region	<i>Trichphyton</i>	<i>M. gallinae</i>	<i>M. gypseum</i>	<i>Exserohilum</i>	<i>Acremonium</i>	<i>A. niger</i>
<i>E. coli</i> M2	El-Madina	45	72	85	85	75	15
<i>B. megaterium</i> M3	El-Madina	70	69	60	75	85	25
<i>B. subtilis</i> M10	El-Madina	65	85	67	65	60	24
<i>B. subtilis</i> T2	El-Taif	20	0.0	0.0	45	0.0	0.0
<i>B. subtilis</i> T3	El-Taif	0.0	0.0	0.0	0.0	0.0	12
<i>B. subtilis</i> J1	El-Jouf	60	85	40	75	12	20
<i>B. subtilis</i> J3	El-Jouf	50	22	40	35	70	20
<i>B. subtilis</i> D2	El-Dammam	30	50	0.0	50	12	15
<i>B. subtilis</i> D4	El-Dammam	25	57	45	50	30	0.0

Table 2. Effect of different antibiotics on the growth of *E. coli* and *Bacillus* isolates.

Strain code	Sm 10	Tc 30	Nor 10	Amp 25	Gm 10
<i>E. coli</i> M2	S	S	R	R	S
<i>B. megaterium</i> M3	S	R	R	R	S
<i>B. subtilis</i> M10	S	R	S	R	S
<i>B. subtilis</i> J3	S	S	S	S	S
<i>B. subtilis</i> T2	S	R	S	R	S
<i>B. subtilis</i> T3	S	R	R	S	S
<i>B. subtilis</i> D4	S	R	S	R	S
<i>B. subtilis</i> D2	S	S	S	S	S
<i>B. subtilis</i> M3D3	S	S	S	S	S

S, Sensitive; R, resistant; Sm, streptomycin 10 µg; Tc, tetracycline 30 µg; Nor, norfloxacin 10 µg; Amp, ampicillin 25 µg; Gm, gentamycin 10 µg.

Staphylococcus aureus (MRSA). *E. coli* M2 effectively suppressed colony growth of all tested fungal species. Yadav et al. (2010, 2007) reported that cytosolic proteins of *E. coli* are responsible for antifungal potential against pathogenic strains of *Aspergillus fumigatus*, *Aspergillus flavus*, *A. niger* and *Candida albicans*. Also, Mushtaq et al. (2010) reported that *E. coli* showed almost complete inhibition against pathogenic fungi such as *A. niger* and *A. flavus*.

Antibiotics response of *Bacillus* and *Escherichia coli* isolates

Five antibiotics were used to test all *Bacillus* and *E. coli* strains for their antibiotic response. Data are shown in Table 2. The results show that all isolates were sensitive to Gm and Sm. At the same time, *B. subtilis* (M3D3), *B. subtilis* (J3) and *B. subtilis* (D2) were sensitive to the five antibiotics, and *B. megaterium* (M3) was resistance to Nor, Tc and Amp. The nine isolates were genetically different in their patterns of antibiotic resistance except *B. subtilis* (M10) which resembled *B. subtilis* (T2) and *B.*

subtilis (D4) resistance pattern. *E. coli* (M2) was resistance to Amp and Nor, and *B. subtilis* (T3) was resistance to Tc and Nor. The susceptibility of bacilli to different antibiotics has been studied previously, and it has been demonstrated that, in principle, it should be possible to identify species on the basis of the results of susceptibility tests (Burke and McDonald, 1983; Castro et al., 1991). A large number of *Bacillus* strains assigned to different species were tested to determine their susceptibilities to antibiotics. Considerable interspecific differences in susceptibility were observed with chloramphenicol, Amp, and Tc (Reva et al., 1995).

Molecular characterization of the bacterial isolates by PCR

Studying genetic diversity of natural bacterial isolates as a new biocontrol agents became a global programs to determine genetic variation among strains populations. Two primers were selected to be used in this study. In order to score fingerprints, one band has assumed to be corresponded to one locus. A total of 148 allelic bands

1 2 3 4 5 6 7 8 9 10 11

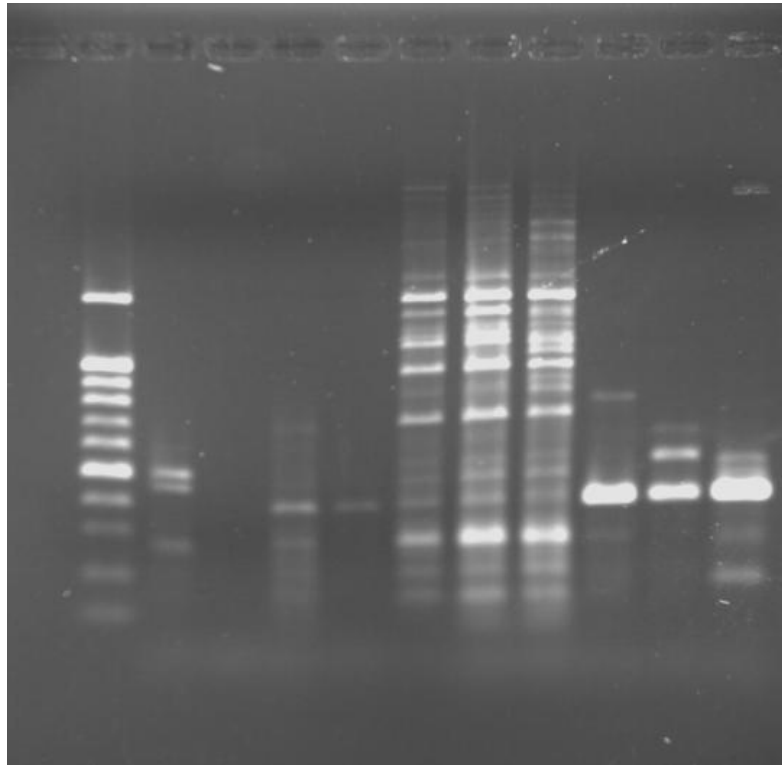


Figure 1. Photograph of DNA amplified banding patterns based on RAPD for ten different strains: *E. coli* M2, *B. megaterium* M3, *B. subtilis* J3, *B. subtilis* T2, *B. subtilis* D4, *B. subtilis* D2, *B. subtilis* M3D3, *B. subtilis* T3, *E. coli* pUC19 and *B. subtilis* M10 (lanes 2, 3, 4, 5, 6, 8, 9, 10 and 11) against 100 bp ladder DNA marker which has three distinct bands: 500, 1000, 2000 bp (lane 1) using primer No.1.

ranging between 100 and ~3000 bp were amplified as shown in Figures 1 and 2 for primers 1 and 2, respectively. No band out of 148 bands was monomorphic in all strains. The RAPD profiles of the ten strains were compared, and variation in the band profiles was observed for each primer; each strain has specific bands which were absent in others. The highest number which corresponded to 21 loci was found in *B. subtilis* M3D3, with primer No. 1, which ranged from 100 bp to ~3000 bp. The lowest number which corresponded to 1 locus was found in *B. subtilis* T2, with primer No. 1, which was 400 bp band. Analysis of the nine isolates by using two RAPD primers revealed that a total of 148 bands were scored (Table 3). *B. subtilis* D4 and *B. subtilis* D2 isolates were closest to *B. subtilis* M3D3, which possessed moderate antifungal activity; was found to be very diverse from *B. subtilis* M10 which had the most efficient antifungal activity. This diversity is highly correlated with habitats and the antagonistic effect of the

studied strains.

DNA markers indicate the differences in the sequences of nucleic acid at a particular location or locations in the genome. In addition, they are independent of external factors, and the rates of polymorphism in these variable regions are high (Parmaksız, 2004). One of the DNA marker techniques, RAPD, is based on the amplification of unknown DNA sequences and uses a single short and random oligonucleotide primer (Williams et al., 1990). This technique, which is fast and inexpensive, can detect DNA variations even at a single-base level. Hence, it has become a commonly used technique for exploring intraspecies and interspecies diversity (Roy et al., 1992; Carlson et al., 2001; Bardakc, 2001; Arslan and Tamkoc, 2011).

It was reported that high metabolic activities of *Bacillus* strains often correspond to their specific genotypes (Pinchuk et al., 2002). This genetic heterogeneity is interesting in view of the production of antimicrobial

1 2 3 4 5 6 7 8 9 10 11

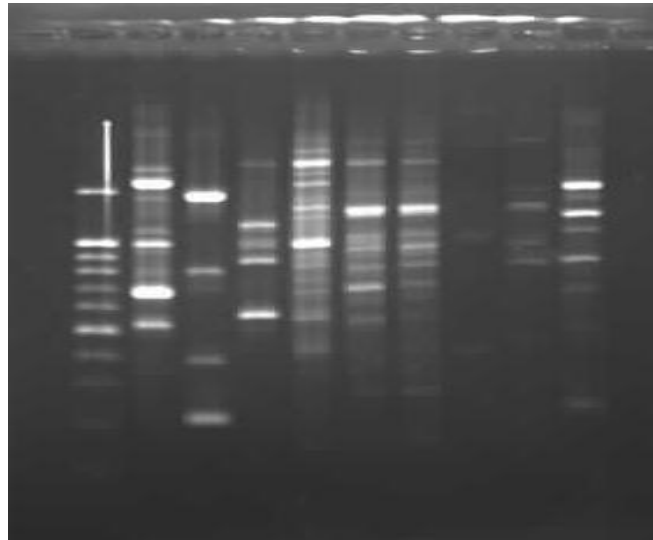


Figure 2. Photograph of DNA amplified banding patterns based on RAPD for ten different strains: *E. coli* M2, *B. megaterium* M3, *B. subtilis* J3, *B. subtilis* T2, *B. subtilis* D4, *B. subtilis* D2, *B. subtilis* M3D3, *B. subtilis* T3, *E. coli* pUC19 and *B. subtilis* M10 (lanes, 2, 3, 4, 5, 6, 8, 9, 10, and 11) against 100 bp ladder DNA marker which has three distinct bands: 500, 1000, 2000 bp (lane 1) using primer No.2.

Table 3. Number of DNA polymorphic bands in ten bacteria isolates amplified with two oligonucleotide primers.

Primer number	Bacteria isolate	Number of fragments scored of polymorphic loci
1	<i>E. coli</i> M2	3
	<i>B. megaterium</i> M3	0
	<i>B. subtilis</i> J3	5
	<i>B. subtilis</i> T2	1
	<i>B. subtilis</i> D4	18
	<i>B. subtilis</i> D2	19
	<i>B. subtilis</i> M3D3	21
	<i>B. subtilis</i> T3	3
	<i>E. coli</i> pUC19	3
	<i>B. subtilis</i> M10	5
2	<i>E. coli</i> M2	8
	<i>B. megaterium</i> M3	6
	<i>B. subtilis</i> J3	5
	<i>B. subtilis</i> T2	13
	<i>B. subtilis</i> D4	10
	<i>B. subtilis</i> D2	10
	<i>B. subtilis</i> M3D3	4
	<i>B. subtilis</i> T3	6
	<i>E. coli</i> pUC19	8
	<i>B. subtilis</i> M10	0

activity. Use of the RAPD method has extensive application for rapid typing of microorganisms at the species and subspecies levels (Stephan et al., 1994; Ronimus et al., 1997; Pinchuk et al., 2002). Due to the diversity of the environmental condition, the species or different strains of the same species such as *E. coli* did not show the same sort of phylogenetic relationship among each other. Most of these different *E. coli* RAPD types were not geographically restricted. There was, as expected, a tendency of higher genetic relationship among *E. coli* strains isolated from the same locality, which was finally obtained, while some other strains differed from each other due to the environmental impact (Satyajit, 2009).

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