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RAPD Fingerprinting of Indigenous *Lysinibacillus fusiformis* Isolates from Stabilized Sludge and Oil-Polluted Soil

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ABSTRACT

The *Lysinibacillus* group are motile Gram-positive bacilli, with oval or spherical spores, oxidase and catalase positive, not grouped in strict chains, with strictly oxidative metabolism and very similar to *Bacillus sphericus* group.

Two indigenous isolates of *Lysinibacillus fusiformis* from oil-polluted soil near gas station and stabilized sludge from petrochemical plant in Serbia (FOV – HIP "Petrohemija", Pancevo) were tested on IAR and HMT and showed high sensitivity to neomycin, cephalexin and bacitracin, and were resistant to trimethoprim. Both isolates were tolerant to 100µg/ml of Zn and Co, 10µg/ml of Mo, but they differed in tolerance to 20µg/ml of Cd and 10µg/ml of Hg. The isolate from stabilized sludge showed moderate emulsification ability of xylol (E_{24} 65.8) and mineral oil (E_{24} 75.6). The isolate from oil-polluted soil showed very strong emulsification ability of xylol (E_{24} 87.2) and moderate of mineral oil (E_{24} 72.7). RAPD fingerprinting showed clear differences between the two *Lysinibacillus fusiformis* isolates.

Keywords: *Lysinibacillus fusiformis*, polluted soil, sludge, heavy metals, emulsification ability, RAPD fingerprinting

INTRODUCTION

Mesophilic, strictly aerobic, spore-forming bacilli that are capable of producing spherical endospores have been designated *Bacillus sphaericus*. These bacteria metabolize a variety of organic and amino acids but cannot metabolize sugars, leading to negative results for many of the traditional phenotypic tests used in the classification of members of the genus *Bacillus* (Baumann et al., 1991). The diversity of these bacteria was demonstrated by Krych et al. (1980), who identified five distinct DNA homology groups among the 50 strains using DNA hybridization techniques. Strains belonging to DNA homology group IIA are of particular interest as pathogenic for the larvae of certain species of mosquitoes. The homology IIB group consists of nonpathogenic strains that exhibit 60 to 66% homology with the group IIA reference strain. In a study of 91 *B. sphaericus* strains in which 155 phenotypic tests were used, strains belonging to homology group IIA clustered with strains belonging to homology group IIB at a Jaccard similarity level of 85.5%. In 1988, Priest et al. described species *Bacillus fusiformis,* which is homologue to IIB group according to Krych. Urea-hydrolysing capability

of *Bacillus fusiformis* differentiated it from *B. sphaericus*. Later studies based on random amplified polymorphic DNA (RAPD) probing (Woodburn *et al*., 1995) and ribosomal gene restriction fragment length polymorphism (RFLP) analyses (Aquino de Muro *et al*., 1992) confirmed the separate groupings. Phylogenetic analysis based on 16S rDNA sequences from 58 *B. sphaericus* strains Nakamura (2000) was used to examine the genetic heterogeneity of the taxon. Data showed that *B. sphaericus* was genetically and phenotypically a highly heterogeneous taxon comprised of at least seven genetically distinct taxa, one of which encompassed *B. sphaericus* insect-pathogenic strains and another *B. fusiformis*.

Lysinibacillus fusiformis is described by Ahmed et al. (2007) as spore-forming, Gram-positive, motile, rod-shaped and boron-tolerant (up to 150 mM boron) bacterium isolated from soil, which can tolerate 5 % (w/v) NaCl and has growth range 16–45 °C and pH range pH 5.5–9.5. In contrast to the type species of the genus *Bacillus*, *Lysinibacillus fusiformis* strains contain peptidoglycan with lysine, aspartic acid, alanine and glutamic acid. Comparative analysis of the 16S rRNA gene sequence demonstrated that isolated *Lysinibacillus fusiformis* strains were closely related to *Bacillus fusiformis* DSM 2898^T (97.2 % similarity) and *Bacillus sphaericus* DSM 28^T (96.9 %). DNA–DNA relatedness was greater than 97 % among three isolated strains and 61.1 % with *B. fusiformis* DSM 2898^T and 43.2 % with *B. sphaericus* IAM 13420^T. Based on the distinctive peptidoglycan composition, phylogenetic analyses and physiology, the strains were assigned to a novel species within a new genus, for which the name *Lysinibacillus boronitolerans* gen. nov., sp. nov. was proposed. It was also proposed that *Bacillus fusiformis* and *Bacillus sphaericus* be transferred to this genus as *Lysinibacillus fusiformis* comb. nov. and *Lysinibacillus sphaericus* comb. nov., respectively (Ahmed et al., 2007).

The aim of this study was to characterize and compare the two *Lysinibacillus fusiformis* strains indigenous to oil-polluted soil and stabilized sludge from petrochemical plant in Serbia.

METHODS

Indigenous isolates of *Lysinibacillus fusiformis* from oil-polluted soil (marked BZi) near gas station (BZi4) and stabilized sludge (BMi) from petrochemical plant in Serbia (FOV – HIP "Petrohemija", Pancevo) (BMi3) and *Bacillus sp.* isolates, BMi 9 and BMi12, were tested and compared to isolates BZi1 from oil-polluted soil near gas station, previously identified as *Bacillus sp*. The isolates were collected by the Institute of Soil Science, Belgrade, Serbia. Working stock cultures were incubated at 28 °C on nutrient agar amended with 5 mg $MnSO_4$x $2H_2O$ until sporulation occurred and then stored at 4 °C. Isolates were tested on nutrient agar (NA) supplemented with different antibiotics: novobiocin, neomycin, cephalexin, bacitracin, trimethoprim and clindamycin. Isolates were tested to 100 and 200µg/ml of Zn and Co, 10and 50µg/ml of Mo, 4,10 and 12 µg/ml of Hg and 20µg/ml of Cd added to NA. Emulsification ability of xylole, toluene and mineral oil was assessed as described by Cooper and Paddock (1983). Substrate utilization was tested on BH medium with 1% of toluene and xylole and 0.5% mineral and crude oil (Toledo et al., 2006). RAPD analysis was

performed as recommended by Dooley et al. (1993). Similarity was estimated by means of the simple matching coefficient (*S*SM) and clustering was based on the unweighted pair group arithmetic average-linkage algorithm (STATISTICA 7 software).

RESULTS and DISCUSION

Two indigenous *Lysinibacillus fusiformis* isolates from stabilized sludge (BMi3) and oil polluted soil (BZi4) were assessed for antibiotic resistance and heavy metal tolerance and compared to *Bacillus sp*. (BMi9 and BMi12) from stabilized sludge and *Bacillus thurangiensis* (BZi1) from polluted soil. The results are summarized in Table 1. Isolate BMi3 was more sensitive to novobiocin and bacitracin than other isolates, except for BZi1 which was very sensitive to all investigated antibiotics. BMi3 isolate was tolerant to Cd (20 µg/ml), but showed low tolerance to Mo, Zn, Co and Hg. Isolates BZi4, BMi9 and BMi12 showed high tolerance to heavy metals, especially BMi12, which was tolerant to Hg 12 µg/ml.

Isolate	antibiotic (µg/ml)						heavy metal (µg/ml)						
	Nov 5	Clin 2	Neo 120	Bac 40U	Ceph 30	Tmp 5	Mo 50	Zn 200	Cd 10	Cd 20	Co 200	Hg 2	Hg 10
BMi3	S	I	S	S	S	R	-	-	+	+	-	+	-*
BZi4	I	S	S	I	S	R	+	+	+	-	+	+	+
BZi1	S	S	S	S	S	I	+	+	+	-	+	+	+
BMi9	R	S	S	I	S	R	+	+	+	-	+	+	+
BMi12	R	S	S	I	S	R	+	+	+	-	+	+	+**

-* - isolate sensitive to 4 µg/ml Hg; +**- isolate tolerant to Hg 12 µg/ml
R- resistant, S- sensitive, I- intermediate

Table1. Antibiotic resistance and heavy metal tolerance of *Lysinibacillus fusiformis* and *Bacillus sp.* isolates

Isolate	substrate utilization				emulsification activity		
	toluene 1%	xylole 1%	mineral oil 0,5%	crude oil 0,5%	toluene	xylole	mineral oil
BMi3	+	+	-	-	75.64	65.79	75.61
BZi4	+	±	-	±	92.68	87.18	72.74
BZi1	-	+	+	+	74.42	62.50	78.25
BMi9	±	±	±	+	95.45	99.72	78.40
BMi12	+	+	+	+	97.67	83.82	89.62

Table2. Substrate utilization and emulsification activity of *Lysinibacillus fusiformis* and *Bacillus sp.* isolates

Large number of *Bacillus* strains, including *B. fusiformis*, capable of degrading different hydrocarbons have been isolated from oil-contaminated soils (Bento et al., 2003). Our investigation

showed no growth of isolate BZi1 on toluene, but good growth on other substrates (Table 2). None of *Lysinibacillus fusiformis* isolates were able to grow on mineral oil as only source of carbon, while isolate BZi4 grew poorly on crude oil and xylole and well on toluene. *Bacillus sp*. isolate BMi9 grew poorly on all substrates except crude oil, while BMi12 showed ability to utilize all examined substrates.

Microorganisms growing on hydrocarbons frequently produce biopolymers with emulsifying or surfactant activity (Ron and Rosenberg, 2002), that can facilitate the availability of hydrophobic substrates. Emulsifying biopolimers can stimulate growth of hydrocarbon degrading bacteria and improve their ability to utilize these compounds (Toledo et al., 2006). In this study, the capacity of isolates to produce extracellular biopolymers with bioemulsifier activities was assayed. Emulsification activity of *Lysinibacillus fusiformis* isolate BMi3 ranged from 65.79 (xylole) to 75.64 (toluene), and it was lower for BZi4 isolate (72.74 for mineral oil and 92.68 for toluene). Isolate BZi1 from polluted soil was the least effective, while *Bacillus sp.* isolates BMi9 and BMi12 from activated sludge were highly effective in emulsification, especially BMi9 on xylole (99.72) and BMi12 on toluene (97.67). Previous reports on different microorganisms showed variable values of emulsification of hydrocarbon by cell-free broth after 24h: 10-70 *Torulopsis petrophilum* (Cooper and Paddock, 1983), 25-68 *Bacillus sp*. IAF343 and 70 *B.cereus* on pH 2-7 (Cooper and Goldenberg, 1987). E_{24} value for *Lysinibacillus fusiformis* isolates from this work were similar to previously reported, except for BZi4 isolate on toluole as investigated solvent that showed higher emulsification values. The two *Bacillus sp.* isolates from stabilized sludge investigated in this work also showed high emulsification activity, suggesting that they produced high amount of biopolymers that act as good emulsion stabilizers.

The RAPD method is very useful in fingerprinting of bacteria because previous sequence information is not necessary (Williams et al., 1990). Woodburn et al. (1995) used the ability of RAPD fingerprinting data to indicate heterogeneity within the *B. sphaericus* isolates. RAPD analysis in this work was performed to confirm differences between two *Lysinibacillus fusiformis* isolates and to determine genetic distance of investigated isolates. Results of genotypic analysis (Figure 1) and similarity level are shown as dendrogram in Figure 2.

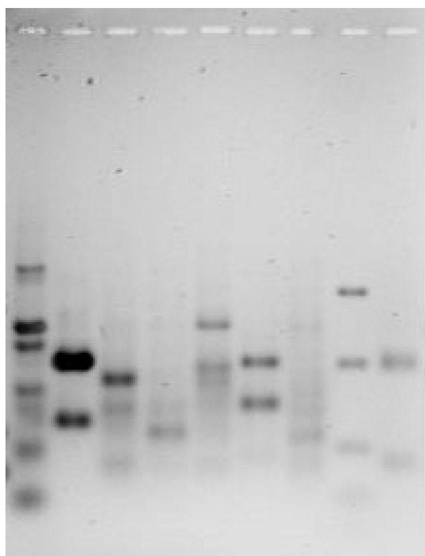

Figure 1. RAPD band patterns generated by using SPH1 primer and DNAs from indigenous *Lysinibacillus fusiformis* (lane 1- BMi3 and lane 2- BZi 4) and *Bacillus sp.* isolates (lane 3- BMi9; lane 6- BMi12 ;lane 9- BZi1). Marker: lane 8 -FastRuler Low Range DNA Ladder SM1103 (Fermentas) with 1500; 850; 400 and 200 bp bands

The results of RAPD fingerprinting support the results of phenotypic analysis that two *L. fusiformis* isolates are quite different. The similarity level between them obtained with SPH1 primer was only 30%. *Bacillus sp*. isolates formed a cluster with 78% of similarity. BZi4 isolate showed highest similarity level to *Bacillus* group, then to *Lysinibacillus fusiformis* BMi3. Anandkumar and Maruthamuthu (2008) who investigated manganese oxidizing strains from orthodontic wires by 16S rRNA sequencing, described two *Lysinibacillus boronitolerans* strains that were phylogenetically diverse from *Lysinibacilus fusiformis* strain.

Genotypic and phenotypic analysis showed significant differences between two *Lysinibacillus fusiformis* isolates investigated in this study. Ability to tolerate heavy metals, to grow on toluene, and to produce a biopolymer with high emulsification activity gives the isolate BZi4 potential for use in bioremediation process. *Bacillus* isolates BMi9 and BMi12 could also potentially be used in bioremediation.

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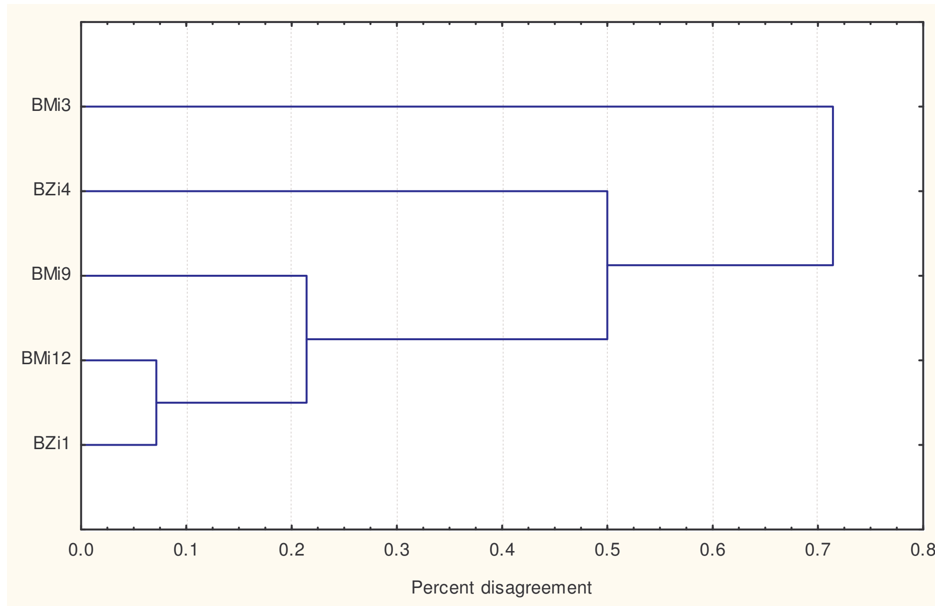

Figure 2. Dendrogram of RAPD generated by SPH1 primer on *Lysinibacillus fusiformis* and *Bacillus sp.* isolates