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Isolation and characterisation of lead-resistant bacteria from a reverse screen model of simulation soil rice culture

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Abstract. CGMCC5515(Rif^r) were domesticated step by step. Pb-resistant bacteria (PbRB) of CGMCC5515 were selected in directional enrichment and screened with TTC from the pot rice rhizosphere soil with lead and other remediate material, the reverse screen model of simulation soil rice culture with reproducible TTC-detective method could be used as a tool in the R & D of bioremediate agent of lead-polluted soil. The PbRB with lead tolerance 1500 mg L^{-1} were identified as *B. subtilis* on the basis of morphological, biochemical and partial sequencing of their 16S rRNA and *gyrB* genes. Multiple stress tolerant CGMCC5515 immobilizated in alginate beads, formulated with biochar, attapulgite humic acid, reduced the pb of rice seeding to normal level. These results have reinforced the biotechnological potential of CGMCC5515 as a biological control agent.

INTRODUCTION

The remediation of heavy metal contaminated farmland is a global challenge. Environormental problems in soil, water and underground water have impaired the quality of agricultural foods, damaging human health.

Slight contamination could be remediated by agronomic regulation technology. A moderate one can be tackled with through soil passivation, leaf resistant control, and water management. For heavily contaminated farmlands, crops are normally no longer planted and phytoremediation is mostly used. Microorganmism can be helpful when used in all situations above. Because of being highly efficienct, economic, green, clean and environmentally friendly, microbial remediation precluding heavy metals from entering the biosphere[1-3] raises more concern in heavy metal pollution treatment. Economy is an additional factor to assess the effectiveness of heavy metals remediation, so unless in situations of severe pollution, the fertilizer against heavy metals pollution will normally be well received. Organic fertilizers, microbial fertilizers as well as compound microbial fertilizers, which immobilizate lead, cd and etc, are all good choices.

Heavy contaminated farmland mostly uses the phytoremediation without crop production. Microorganism may be helpful for all the contamination above. Because of being high efficiency, economy, green, clean and environment-friendly, microbial remediation which could preclude or

immobilizate heavy metals from entering the plant, play a important role in heavy metal pollution farmland treatment and pollution ecology.

Economics is an additional factor to assess the availability of heavy metals remediation technology. Therefore, fertilizer for heavy metals pollution remediation has been well received unless severe pollution prevents planting. Organic fertilizer, microbial fertilizer and compound microbial fertilizer, which could immobilizate lead, cd, etc, are all good choices.

Plant Growth-Promoting Rhizobacterial (PGPR) colon in the rhizosphere (the root-soil interface), which is a site of intense interplay between plant and microorganisms. As an ecological niche, the rhizosphere is characterized by plant-based selection, interplay for space and nutrients and strees. CGMCC5515 belong to PGPR, and the compound microbial fertilizer with biological pesticide function made by CGMCC5515 [4] has been put into production in China.

The *gyrB* , encoding subunit protein of DNA gyrase , a typeII DNA topisomerase, commonly found in bacteria. Although the similarity of gyrB gene sequence between different strains of *Bacillus subtilis* is very high, phylogenetic tree could still determine the genetic relationship among different strains. Phylogenetic tree obtained by16S rDNA / RNA and *gyrB* genetic information can rapidly and accurately identify *Bacillus subtilis* in species level [5].

TTC, the reaction of succinate dehydrogenase with TTC lead to the formation of red three phenyl armour (TTF), principle of which is similar to MTS (3-(4,5-dimethyl-2-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium bromide) assay[6]. Appropriate reverse selection strategies for microbial remediation of heavy metals in farmland are chosen. PbRB(Pb-resistant bacteria) of CGMCC5515 mixed with biochar, attapulgite humic acid can make the rice seeding containing pb 500 mg/kg in soil reduce to 0.1863 mg/kg of rice seeding, reaching the Limit of pollutants in foods (GB 2762—2012China), at same time, isolated and identified of lead-resistant bacteria from a reverse screen model of simulation soil rice culture in this study.

MATERIALS and METHODS

Rifampicin resistance marker

The activated *Bacillus subtilis* was inoculated into culture medium containing rifampicin. The concentration of rifampicin increased gradually as follows: in a shake flask of 20mg/mL rifampicin→40mg/mL rifampicin; sterile shake flask→40mg/mL rifampicin to 100mg/mL rifampicin. Rifampicin-resistant strains serially passed 30 times with one subculture with rifampicin and another subculture without rifampicin. The mutant bacteria of domestication are highly efficient for screening mutant of rifampicin-resistant.

Reverse screen model of simulation soil rice culture

The bacterium was cultured and prepared as a stock suspension of endospores according to the method described by S.P.XUE et al. [6]. Formulations of alginate microcapsules were prepared, with endospores of CGMCC5515(Rif^r) using an ionotropic gelation method, which mixed a 4 % (w/v) sodium alginate solution. The alginate-bacterium mixture (50 ml) was magnetically stirred for an hour and then extruded through an injector into $CaCl_2$ (0.5 M, 100 ml) to form microcapsules. The calcium alginate microcapsules were washed with sterile distilled water, and inoculated into the pot with pb(500mg/kg) for six months. Rice seeding were transplanted at trefoil stage, and grow for 2 month, and then random rhizobacterial soil were collected from rice seeding root.

Pb-resistant bacteria screened by TTC

The PbRB were isolated from rhizophere of rice by media with rifampicin (100mg/mL) and pb (1500mg/kg). Growing colon were selected and preserved after streak purification for three times.

To determine the maximum tolerance concentrations (MTC) of PbRB, the resistant isolates were inoculated into 5 mL of MH broth supplemented with different pb concentration (100, 200, 400, 600, 800, 1200, 1300, 1400, 1500, 1600 mg L^{-1}), respectively. The cultures were incubated at 28°C for 2 days, and then added 0.1mg/L of TTC. TTC is the indicator for the quantification of microbial growth in microplates with pb. It is better to add TTC after bacteria completed the growth, and OD600 were tested half an hour after adding TTC later by enzyme-labelled meter(HBS-1096A).The highest pb concentration at which the bacteria could grow was designated as the MTC[7].

1.4 Bacteria identification

Morphological, Physiological，biochemical identification of bacteria were carried out using Biolog GenIII Microstation (USA) identification system (hereafter, GEN III）according to the program reported by P Wragg [8]. 16S rRNA were amplified by PCR using the following primers 27 f (50-GAGTTTGATCACTGGCTCAG-30) and 1492r 50-TACGGCTACCTTGTTACGACTT-30). Amplification was performed for 30 PCR cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min. The amplified DNA was purified with TaKaRa Agarose Gel DNA Purification Kit (TaKaRa, China) and sequencing was performed at TaKaRa Biotechnology Company, Limited (Dalian, China). The 16S rRNA sequence（1500bp） was compared against the GenBank database using the NCBI Blast program.

The genotype of the *gyrB* genes were amplified by PCR as described previously (J Yoon,2010) [9]. A phylogenetic tree was created through cluster analysis by MEGA7.0.

1.5 Analytical technique

The concentrations of heavy metal ions were determined by the graphite furnace atomic absorption spectrometry analyses using atomic absorption spectrophotometer (Puxi, China).The hollow cathode lamp was operated at 5 mA and the analytical wavelengths were set at 228.8 for the detection of Pb (II). Samples were prepared according to China national standards for food safety - Limits of pollutants in foods (GB2762-2012) .

Results and Discussions

Screening pb-resistant bacteria

After two months cultivation in reverse screening soil simulation modle of rice culture, MTC of CGMCC5515 increases from 100 mg/kg to 1500mg/kg. Reverse screening is effective for the stress tolerant domestication in simulating polluted farmland system. The exposure to toxic substances such as heavy metals for a certain period could encourage the natural selection of a resistant population in this study. TTC could inhibit bacteria in high concentration, the TTC's MTC for CGMCC5515 is 1 mg/L(100μg%)seen in red (Fig.1), Therefore, the experiment was conducted with 0.1 mg/kg TTC. It is better to add TTC after bacteria complete the growth, and test OD_{600} half hour later.

Fig.1 MTC of TTC in CGMCC5515 (0: control,100: 100μg% TTC)

Identification of pb-resistant bacteria

The results of physiological and biochemical characters of PbRB have been shown in Table1. These studies have shown that strains resistant to one stressor could withstand most stresses existing in their environment. The results of resistance of PbRB saw in Table2 show resistant to salt, K_2O, antibiotics, aztreonam, sodium butyrate, LiCl, Rifomycin, guanidine, sodium bromate and butyrate. The growth experiment of PbRB had been done in about 63 kinds of chemical matter, as shown in Table3.

Table 1 The results of physiological and biochemical characters of PbRB

Test item	result	Test item	result	Test item	result	Test item	result
cellular morphology	straight rod	Gram stain	Posiytive	Spore	+	Cyst enlargement	-
Ph6.0	+	50°C	+	Indole	-	4%NaCl	+
Ph5.0	-	VP	+	Urease	-	Gelatinase	+
1%NaCl	+	MR	-	H2S	-	catalase	+
Arginine digydrolase	+	Nitrate reduction	+	Ornithine decarboxylase	-	lysine decarboxylase	-
8%NaCl	+	Citrate	+	galactosidase	+		

Table2 The results of resistance of PbRB

Test item	result	Test item	result	Test item	result	Test item	result
positive control	+	Rifomycin SV	+	lincomycin	-	fusidic	-
1%sodium lactate	+	lithium chloride	+	guanidine hydrochloride	+	Potassium tellurous acid	+
minocycline	-	D-serine	-	troleandomycin	-	aztreonam	+
sodium tetradecyl sulfate	-	sodium bromate	+	vancomycin	-	sodium butyrate	+
tetrazolium violet	-						

Table3 The results of PbRB growth in 63 chemical maters

Test item	result	Test item	result	Test item	result	Test item	Test iten
Negeative control	-	bromosuccinic acid	-	L-galacturonic acid lactone	+	D-galacturonic acid	+
D-maltose	+	D-fructose	+	D- sorbitol	+	L-fucose	-
D-trehalose	+	D-salicin	+	D-fucose	-	D-mannose	+
3-methyl-D-glucose	-	D-methyl lactate	-	Methyl D-glucoside	+	L-pyroglutamic acid	-
a-D-glucose	+	a-D-lactcose	-	D-galactose	-	D-cellobiose	+
		propionic acid	-	aminobutyric acid	-	D-mannitol	+
turanose	+	D-malic acids	-	L-malic acids	+	Gelatin	+
inositol	+	glyceol	+	mucic acid	+	saccharic acid	-
formic acid	+	acetic acid	-	L-lactic acid	+	sucrose	+
α-ketobutyric acid	-	D-gluconic acid	+	α-etoglutaric acid	-	N-acetyl neuraminic acid	-
Citric acid	+	L- alanine	+	L-aspartic acid	+	L-glutamic aci	+
L-arginin	-	L-histidine	+	L-serine	-	acetacetic acid	-
D-arabitol	-	Tween40	-	D-aspartic acid	-	ketobutyric acid	-
α-hydroxybutyric acid	-	Hydroxy DL-butyric acid	-	N-acetyl D-galactosamine	-	N-acetyl D-mannosamine	-
gentiobiose	+	turanose	+	quinic acid	-	dextrin	+
gentianose	+	D-raffinose	+				

phylogenetic analysis using16 S rRNA and gyrB sequence

The 16S rRNA sequences of this strain were used to search the GenBank (NCBI). The *gyrB* sequences of this strain as query were used to search the GenBank (NCBI). The query sequences and the homologous sequences of 16 S rRNA and gyrB sequence, respectively, were aligned using ClustalW and a phylogenetic tree was created in MEGA 7.0 software using the NJ method, as shown in Figure 2. It is suggested that CGMCC5515 is one kind of taxa species in B.subtilis group,

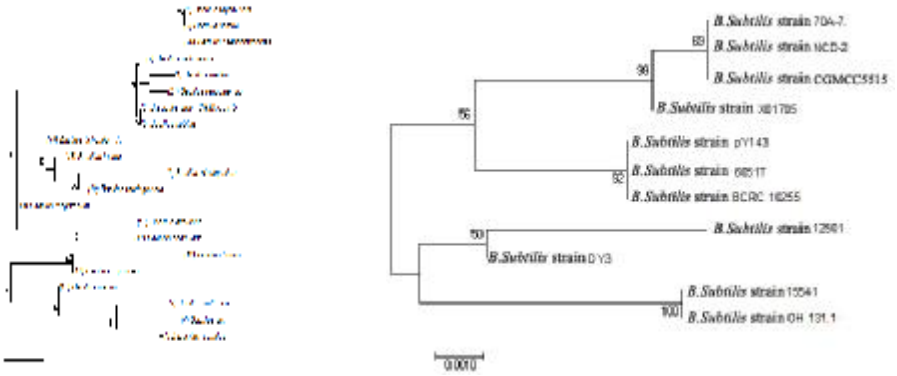

Fig.2 Phylogenetic trees, based on 16S rRNA (left), gyrB gene sequences (right) of the CGMCC5515 and their closest relatives

according to phylogenetic tree result. The morphological, staining, biochemical (Table 1-3) and 16 S rRNA and *gyrB* sequencing analysis of PbRB were highly closest matches being B.

Subtilis[10].The *gyr B* gene sequences provide higher resolution than 16S rRNA gene sequence.[11] 0.1863 mg/kg of rice seeding pb in pot soil of 500mg/kg pb, reaching the national Lead limits standard in China, which benefit from synthetical effect such as biochar, attapulgite and humic acid, beside PbRB[12-14].

Field remediation experiment has been influenced by multiple factors such as weather, soil, and hydrology, different from stimulate pot experiment. ***B. subtilis*** CGMCC5515 will be put into field experiment of the farmland pb remediation as soon as possible.

Conclusions

CGMCC5515 could be multi stresses resistant, belong to ***B.Subtilis***, which is species level, ***gyrB*** is more accurate than 16S rRNA, ***gyrB*** gene may be powerful alternative target for identification and taxonomic analysis of members of the ***Bacillus subtilis*** group.

The TTC-detective and reverse screening soil simulation model may be used as a tool in the R &D of bioremediate agent of lead farmland pollution.

Combination with biochar, attapulgite, humic acid, ***Bacillus subtilis*** CGMCC5515 remediate pb 500 mg/kg in soil to 0.1863 mg/kg of rice seeding, need experiment in field and theory study urgently.

Our results would reinforce the biotechnological potential of strain ***Bacillus subtilis*** CGMCC5515 as a remediation agent for pb polluted farmland.

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