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Evidence of The Presence of Bacteria Highly Resistant to β-Lactam Antibiotics in Taklimakan Desert and Biochemical Characterization of *Paramesorhizobium deserti* gen. nov., sp. nov.*

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Abstract During the surveying of indigenous bacterial diversity in Taklimakan Desert of Xinjiang of China, we obtained 10 isolates, which possess a capacity to grow on 0.1×Tryptic Soy Broth agar supplemented with 1000 mg/L ampicillin. These bacteria display extensive resistances to different β-lactam antibiotics. All of these isolates belong to *Proteobacteria*. Five of them were identified as the human opportunistic pathogen *Stenotrophomonas matltophila*. Four strains were closely related to *Mesorhizobium amorphae*. It is interesting to see that one isolate A-3-E^T showed low 16S rRNA gene sequence similarity (< 96.8%) to those of the recognized species. The results of polyphasic taxonomy showed the strain represents a novel species of the new genus, for which the name *Paramesorhizobium desertii* gen. nov., sp. nov. is proposed. Moreover, it was found that high concentrations of β-lactam antibiotics are not able to inhibit the growth of strain A-3-E^T. The novel bacterium grows well in the media containing 1000 mg/L of cefazolin or 250 mg/L of cefotaxime. The strain also resists 17 of the 28 tested antibiotics. We just presented a case that the Taklimakan Desert is a natural reservoir of novel β-Lactam antibiotic resistant bacteria.

Key words β-lactam antibiotics, antibiotic resistant bacteria, Taklimakan Desert
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The emergence of multi-drug resistant pathogen is a worldwide clinical threat. The study on the origin of antibiotic resistant mechanisms has evoked considerable interest in recent years. It is commonly accepted that the increase of antibiotic resistant pathogens is highly correlated with the clinical use of antibiotics[1–3]. However, antibiotic resistant genes were detected in diverse natural environments including wild rodents' body [4], sponge [5], aquatic environments [6], rhizosphere of wild plant [7], deep sea environments [8], glacier [9] and fresh water [6]. Soil bacteria and human pathogens always share "antibiotic resistome" [10]. This suggests that natural bacterial hosts of antibiotic resistant genes have been severely underestimated. Soil microbiota represents one of the ancient evolutionary origins of antibiotic resistance and has been proposed as a reservoir of resistance genes available for exchange with clinical pathogens [11–12]. Therefore, isolation and identification of novel antibiotic resistant bacteria in different environments that would affect human living environments and the discovery of their mechanisms of resistance probably enables us to better understand the evolution of antibiotic resistance and to find new strategy to minimize their harmful effects to the public health.

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Taklimakan Desert, located in the west of China, is known as one of the largest sandy deserts in the world, and covers an area of 270 000 km^2 of Tarim Basin. We attempted to investigate the possibility of the presence of bacteria with strong resistances to β-lactam antibiotics in this desert. Ten strong β-lactam antibiotic resistant strains were isolated from three surface samples. We determined their susceptibilities to 28 antibiotics. Most of them resist to 8～10 of the β-lactam antibiotics. All these isolates affiliate to the phylum *Proteobacteria*. Polyphasic analysis showed that Strain A-3-E^T, which is highly resistant to diverse β-lactam antibiotics, represents a novel genus and species of *Proteobacteria*, which is referred to as *Paramesorhizobium deserti* gen. nov. sp. nov.

1 Materials and methods

1.1 Isolation of β-lactam antibiotic resistant bacteria from Taklimakan Desert

Several surface sand samples were collected from the Taklimakan Desert of Xinjiang, China (E84° 17.677', N40° 46.031'). Each sample was obtained in sterile tubes before used. The samples were suspended in 0.85% (*w*/*v*) NaCl solution. The suspensions were serially diluted, and plated onto 0.1×Tryptic Soy Broth (TSB, BD Bactto™) agar supplemented with 1000 mg/L of ampicillin and incubated at 30℃. NaCl solution controls were also serially diluted, and plated onto the same media and incubated at 30℃ to exclude air pollution. Several soil samples collected around the desert were employed as parallel controls.

1.2 Detection of β-lactamase activity

Nitrocefin disks were inoculated with a small portion of the culture from TSB agar plates and observed for a change in color from yellow to red. Control strains included *E. coli* DH5α transformed with pUC18 (positive control), and *E. coli* DH5α (negative control).

1.3 Determination of the antibiotic resistant spectrums

Antimicrobial susceptibility test was performed using the Bauer disk diffusion method described by Buczolita[13].

1.4 Analysis of 16S rRNA gene sequence

The genomic DNA was isolated using a bacteria genomic isolation kit (CASarray Co., Ltd). The DNA fragment of 16S rRNA gene was PCR amplified using the primers 27F and 1492R as previously described by Weisburg *et al*. [14]. Purified PCR products were sequenced by Invitrogen Corporation. Identification of phylogenetic neighbors and calculation of 16S rRNA gene sequence similarities were achieved using the EzTaxon-e server[15]. Phylogenetic tree was constructed by the Neighbor-Joining method implemented in the MEGA5 software[16].

1.5 Phenotypic and chemotaxonomic characteristics of Strain A-3-E^T

Cell morphology was examined using the light phase contrast microscopy (Olympus, BX51) and transmission electron microscopy. Samples for transmission electron microscopy were prepared as described by Golyshina *et al*[17]. Motility of the cell was examined by the method with 0.5%(*w*/*v*) soft-agar. Tolerance of the bacteria for NaCl concentrations was determined by growing bacteria in TSB medium (Difco) containing different concentrations of NaCl (0%～6%, *w*/*v*). Bacterial growth was examined at different temperatures (4, 20, 28, 37, 42℃) and at different pH values (5.0～11.0, at intervals of 0.5 pH unit). Oxidase activity was evaluated *via* the oxidation of 1% *p*-amino-dimethylaniline oxalate. Catalase activity was determined by measurements of bubble production after the addition of 3% (*v*/*v*) hydrogen peroxide solution. Enzyme activities and biochemical features were determined using the API kits (API 20NE, API 20E, API 50CH and API ZYM) according to the manufacturers' instruction. DNA G+C content of the bacterium was determined using HPLC (UltiMate 3000, Dionex). Respiratory quinones were extracted and detected by HPLC as described previously[18-19]. For analysis of fatty acid methyl esters (FAMEs), bacteria were cultured on TSB (Difco) agar for 48 h. FAMEs were then prepared and analyzed following the manual of the standard Microbial Identification System 4.2 (MIDI).

1.6 Preparation of growth curve in the presence of antibiotics

Bacteria were grown in the TSB supplemented with different concentrations of ampicilin. Bacteria growth was monitored by measuring the absorbance at 600 nm (A_{600}).

2 Results

2.1 Isolation and identification of β-lactam resistant strains

All colonies were isolated from TSA that contains 1000 mg/L ampicillin. A total of 10 ampicillin-resistant isolates were isolated. Their 16S rRNA genes were

amplified and then sequenced. As shown in Table 1, five strains are clustered with the species *Stenotrophomonas maltophilia*; 4 strains fell into the class α-*Proteobacteria*, and were closely related to *Mesorhizobium amorphae*; another strain A-3-E^T was not able to be affiliated with any of the recognized genus in the class α-*Proteobacteria*. We found all these isolates hydrolyzed nitrocephin more rapidly comparing with the positive control (Table 2). This suggested β-lactamase contribute to the resistant mechanism against the antibiotic.

Table 1 Analysis of the 16S rDNA sequences retrieved from the isolates

Strain	Closest identified relative (accession number)	Similarity/%	Accession no.
γ-*Proteobacteria*			
H-6-A	*Stenotrophomonas maltophilia* ATCC 13617(T)	99.9	KJ491014
H-3-D	*Stenotrophomonas maltophilia* ATCC 13617(T)	99.9	KJ491015
H-5-F	*Stenotrophomonas maltophilia* ATCC 13617(T)	99.9	KJ491016
H-4-C	*Stenotrophomonas maltophilia* ATCC 13617(T)	99.9	KJ491017
A-3-B	*Stenotrophomonas maltophilia* ATCC 13617(T)	99.9	KJ491018
α-*Proteobacteria*			
H-6-B	*Mesorhizobium amorphae* ACCC 19665(T)	98.9	KJ491010
H-6-C	*Mesorhizobium amorphae* ACCC 19665(T)	99.0	KJ491011
A-3-I	*Mesorhizobium amorphae* ACCC 19665(T)	99.0	KJ491012
A-3-J	*Mesorhizobium amorphae* ACCC 19665(T)	98.9	KJ491013
A-3-E^T	*Mesorhizobium robiniae* CCNWYC 115(T)	96.8	KJ491019

Table 2 Antibiotic resistance of the isolates

		Strains									
		H-6-A	H-3-D	H-6-B	H-6-C	A-3-I	A-3-B	H-5-F	H-4-C	A-3-J	A-3-E^T
Antibiotic	Penicillin G	r	r	r	r	r	r	r	r	r	r
	Carbenicillin	r	r	r	r	r	r	r	r	r	r
	Proproctaphlin	r	r	r	r	r	r	r	r	r	r
	Ampicillin	r	r	r	r	r	r	r	r	r	r
	Piperacillin	r	r	r	r	r	r	r	r	r	r
	Kanamycin	r	r	r	r	r	r	r	r	r	r
	Ceftazidine	r	r	r	r	r	r	r	r	r	r
	Furadantin	r	r	r	r	r	r	r	r	r	r
	Lincomycin	r	r	r	r	r	r	r	r	r	r
	Cefuroxime	r	r	r	r	r	r	r	r	r	r
	Cefalexin Ⅴ	r	r	r	r	r	r	r	r	r	r
	Cefran	r	r	s	s	s	r	r	r	s	r
	Ceftriaxone	s	r	s	s	s	s	r	r	r	r
	Erythromycin	r	r	s	s	s	r	r	s	s	r
	Streptomycin	s	s	s	s	s	s	s	s	r	r
	Cefoperazone	s	s	s	s	s	s	s	s	s	r
	Polymyxin B	s	s	s	s	s	s	r	s	s	r
	Vancomycin	s	s	s	s	s	s	s	s	s	s
	Gentamycin	s	s	s	s	s	s	s	s	s	s
	Amikacin	s	s	s	s	s	s	s	s	s	s
	Tobramycin	s	s	s	s	s	s	s	s	s	s
	Ofloxacin	s	s	s	s	s	s	s	s	s	s
	Tetracycline	s	s	s	s	s	s	s	s	s	s
	Ciprofloxacin	s	s	s	s	s	s	s	s	s	s
	Sulfamethoxazole	s	s	s	s	s	s	s	s	s	s
	Chloramphenicol	s	s	s	s	s	s	s	s	s	s
	Norfloxacin	s	s	s	s	s	s	s	s	s	s
	Cephradine	s	s	s	s	s	s	s	s	s	s
β-Lactamase		+	++	+	w	w	+	+	+	+	++

r: Resistant to the antibiotic; s: Sensitive to the antibiotic. ++: Strong positive reactions; +: Positive; w: Weakly positive.

2.2 Determination of antibiotic resistances of the isolates

Twenty-eight antibiotics were employed in the antibiotic susceptivity assay. The results are presented in Table 2. Besides penicillin G, all isolates also act against other β-lactams including carbenicillin, proctaphlin, ampicillin, piperacillin, ceftazidine, furadantin, cefuroxime, and cefazolin Ⅴ. These strains also show strong resistances to kanamycin and lincomycin, which are both aminoglycoside bactericidal antibiotics. Fifty percent of the isolates resist erythromycin, cefaran and ceftriaxone. Two strains A-3-J and A-3-E^T were found to resist streptomycin. Strain A-3-E^T resists simultaneously the antibiotins of cefoperazone and polymyxin B, but other isolates were not capable of doing so. It is likely that Strain A-3-E^T possesses multiple mechanisms against different types of antibiotics.

2.3 Polyphasic taxonomic analysis of Strain A-3-E^T

The 16S rRNA gene of this bacterium is homologous to those of *Phyllobacterium leguminum* LMG 22833^T (96.3%), *Mesorhizobium robinea* CCNWYC 115^T (96.7%), and *Bartonella robiniae* CCNWYC 115^T (96.8%). It suggested that Strain A-3-E^T could be affiliated with any of the three genus or none. It was shown that most *Mesorhizobium* spp. and some *Phylobacterium* spp. are capable of fixing dinitrogen [20–23]. Any nitrogen fixing activities or *nif* genes were not detectable from our isolate (data not shown). Phylogenetic analysis with the strain A-3-E^T and related taxa showed the 16S RNA gene of this novel isolate forms a distinct branch related to those of the genus *Mesorhizobium* and *Phyllobacterium*(Figure 1). This bacterium was likely to represent a novel genus in the family *Phyllobacteriaceae*.

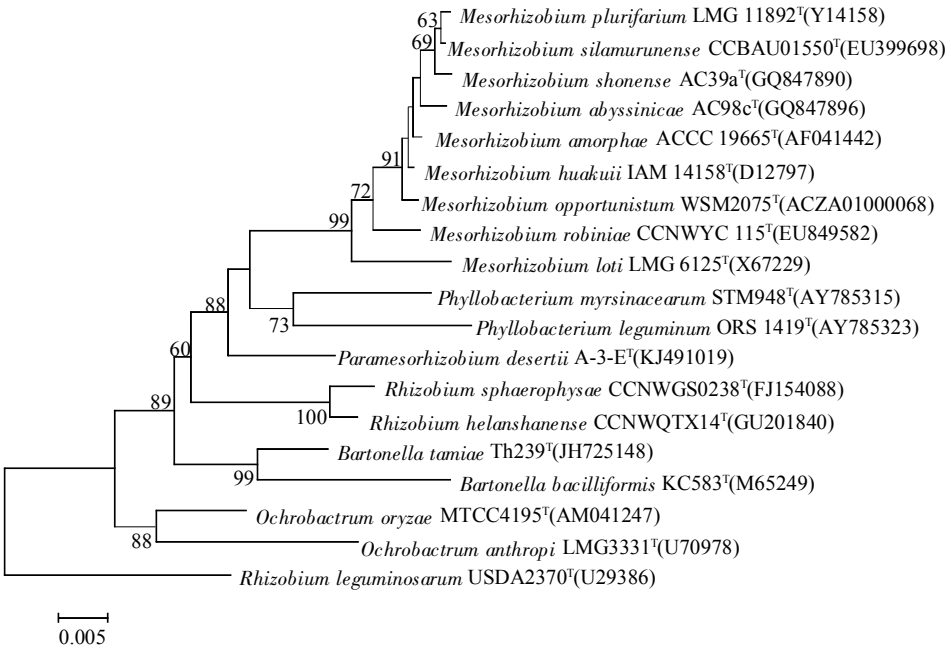

Fig. 1 Phylogenetic tree of A-3-E^T and related species showing the relationships between the new isolate and related taxa

The Bootstrap values (expressed as percentages of 1000 replicates) which are of >70% showing they are at branch points.

The type species of *Mesorhizobium* and *Phyllobacterium* were included as parallel control in phenotypic comparisons. Cells of Strain A-3-E^T was Gram-staining negative, non-spore-forming rods. Single polar flagellum was observed (Figure 2). The colonies were found to be beige, round, and transparent. The strain is catalase- and oxidase-positive. Other features are listed in Table 3, and the descriptions of new genus and species. Several phenotypic features distinguish Strain A-3-E^T from type species of the genera *Mesorhizobium* and *Phyllobacterium* (Table 3). The DNA G+C content of

this bacterium is 60.9% (molar ratio). It possesses quinonine-10 (Q-10) as the respiratory quinonne. This is consistent with those of *Phyllobacterium* sp. and *Mesorhizbium* sp. [24-27]. Strain A-3-E^T differs from *Phyllobacterium myrsinacearum* STM948^T by the lack of $C_{16:0}$ 3-OH and 11-methyl $C_{18:1}$ ω7c and possessing iso- $C_{18:1}$ ω9c. We also distinguished this bacterium and *Mesorhizobium loti* LMG6125^T by the fatty acid variations of 11-methyl $C_{18:1}$ ω7c, iso-$C_{17:0}$, $C_{19:0}$ cycloω8c, and $C_{18:1}$ ω6c (Table 4). Phenotypic characteristics and chemotaxonomic data supported that strain A-E-3^T represents a novel species of a new genus, for which the name *Paramesorhizobium deserti* gen. nov. sp. nov. is proposed. The type strain is Strain A-3-E^T.

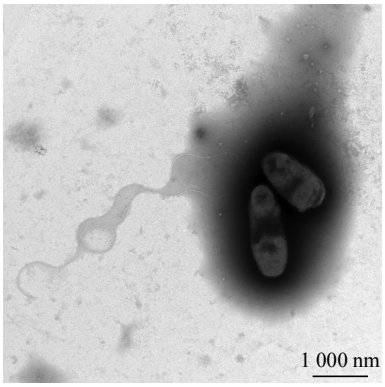

Fig. 2 Transmission electron micrograph of strain A-3-E^T

Table 3 Phenotypic characteristics of strain A-3-E^T and related taxa

Characteristics		*1*	*2*	*3*
Acid from sodium pyruvate		ND	–	+
Assimilation	Potassium gluconate	+	–	w
	D-Ribose	ND	–	w
	D-Saccharose	–	ND	+
	L-Alanine	–	ND	+
	D-Melibiose	–	+	+
	D-Sorbitol	+	–	+
	Valeric acid	+	ND	–
	L-Histidine	–	ND	+
	L-Proline	+	–	+

1: *Phyllobacterium myrsinacearum* STM 948^T; *2*: *Mesorhizobium loti* LMG 6125^T; *3*: Strain A-3-E^T; ND: Not determined.

Table 4 Fatty acid patterns of strain A-3-E^T and related taxa

Fatty acid	*1*	*2*	*3*
$C_{12:0}$ 3-OH	–	Tr	Tr
iso-$C_{13:0}$ 3-OH	–	Tr	Tr
$C_{14:0}$	–	Tr	2.1
iso-$C_{15:0}$	–	Tr	–
$C_{16:0}$	5.0	14.0	16.6
$C_{16:0}$ 3-OH	6.9	–	Tr
$C_{16:1}$ ω7c	2.2	1.4	2.3
$C_{17:0}$	–	2.6	Tr
anteiso-$C_{17:0}$	–	–	Tr
iso-$C_{17:0}$	–	3.5	–
$C_{17:1}$ ω8c	–	1.4	–
$C_{18:0}$	2.6	2.1	8.3
$C_{18:0}$ 3-OH	1.4	–	Tr
11-methyl$C_{18:1}$ ω7c	3.8	14.6	–
$C_{18:1}$ ω6c	65.7	40.2	62.9
$C_{18:1}$ω9c	–	1.9	3.3
$C_{19:0}$ cyclo ω8c	3.8	18.8	1.7
10-methyl $C_{19:0}$	1.2	Tr	–

1: *Phyllobacterium myrsinacearum* STM 948^T; *2*: *Mesorhizobium loti* LMG 6125^T; *3*: Strain A-3-E^T. All data were collected in this study. Tr: Trace (<1%).

2.4 Description of *Paramesorhizobium* gen. nov.

Gram-negative, non-pleomorphic, rod-shaped (1～1.5 μm × 0.6 μm) cells. Form beige, round and translucent colonies (1～3 mm in diameter) on TSB agar incubated at 30℃ for 2～3 days. Oxidase- and catalase-positive. Reduce nitrate to nitrite. Do not fix atmospheric nitrogen. Mesophilic and neutrophilic; pH and temperature for growth are 6.0～8.0 and 20℃～37℃, respectively. Incapable of autotrophic growth. The major cellular fatty acids are $C_{18:1}$ ω6c, $C_{16:0}$ and $C_{18:0}$. The predominant ubiquinone is Q-10. The DNA G+C content is approximately 60% (molar ratio). The genus is a member of the class *Alphaproteobacteria*. The type species is *Paramesorhizobium deserti*.

2.5 Description of *Paramesorhizobium deserti* sp. nov.

Paramesorhizobium deserti (de.ser′ ti. L. gen. n. *deserti* of a desert)

Exhibits the following properties in addition to those given in the genus description: this bacterium

assimilates D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose, malic acid, citrate, L-rhamnose, inositol, D-saccharose, malonate, acetate, lactic acid, L-alanine, 5-ketogluconate, D-melibiose, L-fucose, D-sorbitol, L-histidine, 3-hydroxybutyric acid, 4-hydroxybenzoic acid and L-proline, but not N-acetyl-glucosamine, caprice acid, adipic acid, phenylacetic acid, itaconic acid, suberic acid, 3-hydroxybenzoic acid, salicin, propionic acid, capric acid, and valeric acid. It also positive for producing alkaline phosphatase, esterase (C4), leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, urease, but negative for the activities of lipase (C14), β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, lysine decarboxilase, ornithine decarboxilase, tryptophane deaminase, gelatinase, arginine dihydrolase. It also produces acid from D-fucose, D-xylose, L-fucose, glycerol, erythritol, L-xylose, adonitol, D-galactose, N-acetyl-glucosamine, arbutin, D-salicin, D-maltose, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-arabitol, L-rhamnose, D-melibiose, but not from L-arabinose, D-glucose, D-mannose, L-sorbose, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannoside, methyl-α-D-glucoside, amygdalin, α-D-melibiose, sucrose, D-trehalose, inulin, α-D-melezitose, α-D-raffinose, starch, glycogen, L-arabitol, gluconate, 2-ketogluconate, 5-ketogluconate and D-saccharose. Nitrate reduction and Ⅴ-P test are positive, but indole and H_2S production are negative. Strain A-3-E^T also resists to the antibiotics, penicillin G, carbenicillin, proctaphlin, ampicillin, piperacillin, kanamycin, ceftazidine, furadantin, lincomycin, cefuroxime, cefalexin Ⅴ, cefran, ceftriaxone, erythromycin, streptomycin, cefoperazone and polymyxin B.

The type strain, A-3-E^T, was isolated from the Taklimakan desert of Xinjiang, China.

2.6 Effect of β-lactams antibiotics on the growth of *Paramesorhizobium deserti* A-3-E^T

Bacteria were grown in TSB that contained the drugs or not. As shown in Figure 3, we found that the growth of the culture that contained ampicillin (1 g/L or 8 g/L), or carbenicilin (1 g/L), was similar to that of the control. This indicated that high concentrations of these traditional β-lactams are not able to inhibit the growth of the strain A-3-E^T. We also found that cefotaxime(0.25 g/L) and cefazolin(1 g/L) only slightly inhibited the growth of the strain. These results indicated this bacterium also effectively acted against some cephalosporins.

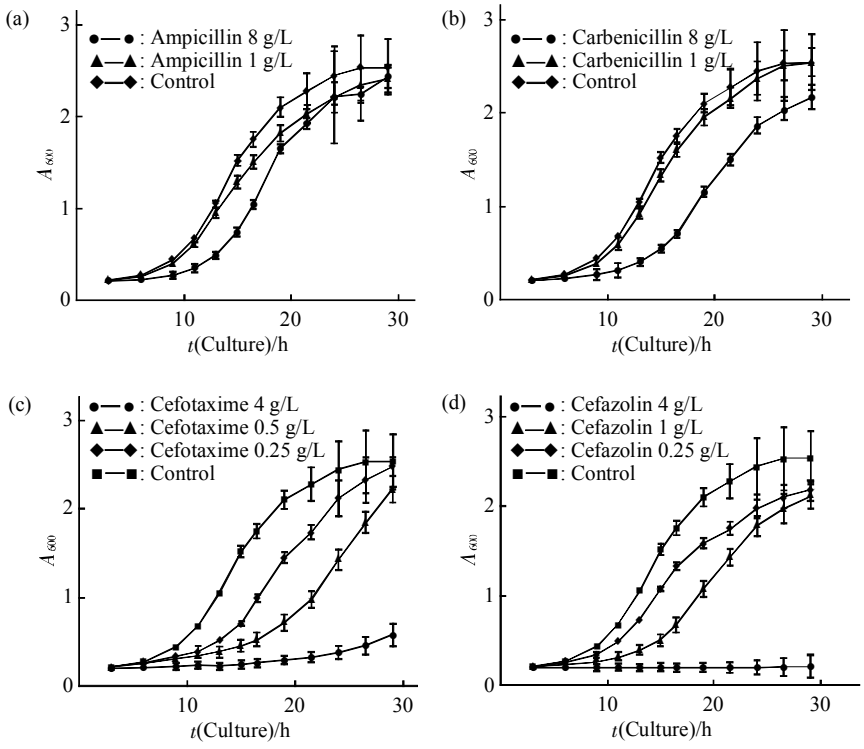

Fig. 3 Effects of β-lactam antibiotics on the growth of strain A-3-E^T

Effects of ampicillin (a), carbenicillin (b), cefotaxime (c) and cefazolin (d) on bacterial growth, which was monitored by measuring the A_{600}.

3 Discussion

Antibiotic resistant mechanisms involve the impermeable barriers, the efflux pumps that pump various toxins, heavy metals, and antibiotics out of cells, the enzymes that degrade or otherwise inactivate antibiotics, and the gene mutations that block or weaken the interaction between the antibiotics and targets [28-29]. Antibiotic resistance genes in human pathogens, such as β-lactamase producing pathogens, have become notorious because they confound the tools that are used to treat disease [30]. The genes contributing to antibiotic resistance in pathogenic bacteria make them more problematic, because of the prevalence of horizontal gene transfer, the process by which bacteria acquire genes from the environment.

What are the natural hosts of the antibiotic resistant genes? To address this issue， isolation and identification of novel antibiotic resistant bacteria would be one of the initial steps to get insight into the unknown antibiotic resistant genes or mechanisms. In this study, we isolated ten strains which were resistant to high concentrations of β-lactam antibiotics from Taklimakan Desert. Five strains showed high homology to the opportunistic pathogen *S. maltophilia*[31]. These desert isolates would be highly homologous to their clinical counterparts. We also found four strains were closely related to *M. amorphae*, suggesting they may inhabit some rhizosphere environment.

We also found novel bacteria species that are highly resistant to β-lactams. Bacteria resistant to high concentrations of one antibiotic drug always possess multiple resistant mechanisms or special enzymes that are able to effectively hydrolyze the substrate. For example, NDM-1, the most famous β-lactamase reported in recent years, is able to hydrolyze diverse β-lactam antibiotics with unsuspected activities[32]. We found that our isolate A-3-E^T is able to hydrolyze nitrocephin suggesting it possess β-lactamase. Unfortunately, we didn't obtain any of the known β-lactamase genes using the PCR strategy (data not shown). This strongly suggests Taklimakan Desert could be a natural reservoir of new antibiotic resistant bacteria, which may possess novel β-lactamase genes or resistant mechanisms. Considering that the sand storm would bring these bacteria to other area and the horizontal gene transfer among a single species or close related species is easy, these bacteria may supply novel antibiotic resistant gene resources to the pathogenic bacteria in other parts of the world.

Bacteria highly resistant to antibiotics are likely to have other primary roles in the environments. For example, it was shown that some soil origin bacteria survive in high concentrations of antibiotics [33-34]. Dantas *et al*. speculated that bacteria can utilize antibiotic as a sole carbon sources, but Walsh *et al*.[35] challenged this concept. Would possessing antibiotic resistance be a strategy for bacteria to inhabit the nutrient poor environment?

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塔克拉马干沙漠存在超耐 β-内酰胺环抗生素微生物的证据及对一个新物种的生物化学特性描述 *

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摘要　在对塔克拉玛干沙漠土著微生物进行研究过程中，发现 10 株细菌能够在含有 1 000 mg/L 氨苄青霉素的 0.1×TSB 平板上生长．这些微生物具有广泛的 β- 内酰氨类抗生素耐受性．它们均属于变形杆菌纲，其中 5 个菌株鉴定为条件致病菌 *Stenotrophomonas matltophila*，还有 4 个菌株与 *Mesorhizobium amorphae* 亲缘关系很近．有趣的是，菌株 A-3-E^{T} 与已知物种同源性低，通过基于生物化学方法的多相分类学研究，我们将其确定为一个新属，并命名为 *Paramesorhizobium desertii* gen. nov., sp. nov.，发现高浓度的 β- 内酰胺类抗生素，如 1 000 mg/L的唑啉头孢菌素或 250 mg/L 的头孢氨噻肟，依然无法抑制其生长．此外，该菌株还能耐受所测试 28 种抗生素中的 17 种，塔克拉玛干沙漠可能是一个新的 β- 内酰胺环抗生素耐药细菌的自然资源库．

关键词　β - 内酰胺类抗生素，抗药细菌，塔克拉马干沙漠
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