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Isolation and identification of two xylanase-producing extremely alkali-tolerant strains of Bacillus halodurans from Turpan in China

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Abstract: Bacillus halodurans XJU-1 and XJU-80 were characterized in terms of physiological and biochemical characteristics, and 16S rDNA sequence homology and DNA-DNA hybridization analysis. The two isolates can grow in nutrient broth at a broad range of pH values from 4.5 to 12.6 for XJU-1 and from 3.8 to 12.8 for XJU-80, respectively. And the optimum temperature of growth were around 39℃ and 42℃, respectively. Phylogenetic analysis of the two strains based on comparison of 16S rRNA sequence revealed that they are closely related to Bacillus halodurans C-125 and DSM497^T with 99% identity. DNA-DNA hybridization showed that the highest levels of DNA-DNA relatedness were found between the two strains (85%) and the B. halodurans type strains (81.3% and 71.5%), respectively. Moreover, the G + C content of the genomic DNA was 40.5 mol% for XJU-1 and 42.2 mol% for XJU-80. Our results demonstrate that strains XJU-1 and XJU-80 should be classified as two new members of the species B. halodurans.

Keywords: Bacillus halodurans; G + C content; 16S rRNA; DNA-DNA hybridization; Phylogenetic analysis

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1 Introduction

In recent years, the study of extremophiles has received attractable research interest. Among the extremophiles the Bacillus genus is one of the most utilized sources for theoretical study and industrial processes such as food, feed, laundry and dishwashing detergent, and paper and pulp industries[1,2] because species of this genus are capable of producing large amounts of enzymes and other products[3]. From this genus, eleven alkaliphilic Bacillus species were taxonomically determined[4]. Due to the usefulness of Bacillus halodurans, several strains (DSM497, Bacillus halodurans C-125, AH-101 and MIR32) had been isolated and identified[4~6]. Based on the importance of the study of Bacillus halodurans, the systematic sequencing of the whole genome of Bacillus halodurans C-125 had been completed in 2000, and now it has been employed as a good model strain[7]. This led to more systematic study of genes and enzymes and the isolation of the alkaliphiles from a variety of environments[8,9]. Many obligate alkaliphilic and halo-tolerant bacteria have been isolated[10]. Several novel genes (ig. the beta-glucanase gene[11], the exo-oligoxylanase gene from Bacillus halodurans C-125[12] the gene of metallopeptidase from Bacillus halodurans H4[13]) had been cloned, expressed and characterized. Meanwhile, differential scanning calorimetric studies of a Bacillus halodurans alpha-amylase have been developed[14]. It has reported that several products of alkaliphilic and halo-tolerant Bacillus strains in 2005, and thus will provide the excellent opportunity to explore novel extremozymes and facilitate identification of the regulatory regions controlling enzyme production in alkaliphilic Bacillus strains.

The XJU-1 and XJU-80 isolates were orignaly isolated from soil in Xinjiang Uyghur Autonomous Region, China. They showed very high alkali-tolerance and were identified as members of the genus Bacillus. In this article, we attempted to taxonomically describe the two strains. Both of the two isolates could produce the xylanase. And the enzymes were found to be active toward xylan at pH12 and the temperature optimum for activity was about 72℃. Recently, xylanase has been found to be applicable in the paper industry, replacing the use of toxic chlorinated species to remove lignin from kraft pulp[15, 16]. And the data of the crude enzymes showed that our present work will facilitate the employments of the two strains.

2 Materials and Methods

2.1 Bacterial strains and methods

The strains XJU-1 and XJU-80 were always done in a beef extract-peptone medium. The pH of the medium was adjusted to approximately 12 by using Na_2CO_3 buffer and by adding NaOH after sterilization. The bacterial strains used in the study are listed in table 2. Bacillus sp. DSM485 and DSM497 were received from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and Bacillus sp. C-125 was a kindly gift from Dr. Hideto Takami from Japan Marine Science and Technology Center, Japan.

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2.2 Physiological and morphological characteristics of the strains

Phenotype tests were performed according to Bergey's Manual of Determinative Bacteriology (7th edition)[17] and the methods described in The Genus Bacillus[18]. Growth and reactions were usually observed after two or three days. Growth and NaCl concentrations of 24% and 26% were recorded for up to 10 days. Hydrolysis of starch and Tweens is observed in the 8th and 14th days, respectively. Reduction of NO^{3-} was recorded for up to 11 days. Indole reaction was tested after seven days. The two strains were inoculated on the medium consisting of (g/L): 2.0g K_2HPO4, 2.0g NH_4NO_3, 0.2g $MgSO_4$, 5.0g yeast extract, 20.0g xylan and 18g agar powder. And the activity of the crude enzyme was determined according to DNS method[19]. The two strains were grown in a beef extract-peptone medium for 24h to check for motility by phase-contrast microscope. Cells were stained according to the classical Gram procedure. For electron microscope, cells were grown in liquid LB medium for 18h to observe the cells. For scanning microscope, cells and spores were treated with 2% uranyl acetate and observed under a Philip PSEM 500 electron microscope. And we can also observe the flagellum and the capsule by transmission electron microscope.

2.3 DNA preparation

Genomic DNA from the strains was prepared using the method described by Saito and Miura (1963)[20]. Briefly, bacterial cells of 2.0mL of each overnight culture were centrifuged at 12000 × g for 5min. The pellets were suspended in 567μL of TES buffer (0.5mol/L Tris-HCl, 0.2mol/L EDTA, 5mol/L NaCl) containing 10% of SDS and 20mg/ml of proteinase K, and then incubated at 38℃ for 1 hour. After phenolization and chloroform purification steps, DNA was precipitated with 2/3 volume of 2-propanol, washed twice with 70% (v/v) ethanol, and dissolved in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH8.0).

2.4 Amplification of 16S rRNA gene and sequence analysis

The 16S rRNA gene was amplified employing primers of 8-27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′)[21]. The PCR products were sequenced employing primer 8-27F or primer 1492R by dideoxy chain termination method using the ABI PRISM377 DNA sequencer. Sequence analysis was conducted with Clustal multiple-alignment program (Clustalx 1.83). Sites involving gaps were excluded from all analyses. A phylogenetic tree was inferred by using the neighbor-joining method[22], using the DNADIST and NEIGHBOR programs in the TREE32 program package.

2.5 Genomic G+C content and DNA-DNA hybridization

The G + C content was determined using thermal denaturation curves[23]. For analysis of relatedness, DNA-DNA hybridization was carried out as described by Christen and Angen (2000)[24].

2.6 Nucleotide accession number and deposition of organism

The DNA sequences of 16S rDNA sequence of strains XJU-1 and XJU-80 have been deposited in GenBank under accession number AY856453 and AY856452, respectively. The two isolates have been deposited as CGMCC 1.4010 and CGMCC 1.4011 in the China General Microbiological Culture Collection Center.

3 Results

3.1 Morphological, physiological and biochemical properties

Taxonomic characteristics of the isolates XJU-1 and XJU-80 are summarized in Table 1. XJU-1 and XJU-80 are all gram-positive, facultatively aerobic, motile, sporulating, straight and rod-shaped (0.8-0.85 × 2.5-5.0μm and 0.7-0.8 × 2.5-5.0μm, respectively) organisms. Sporidia were swollen and central-terminal. The cells of XJU-1 were found to have numerous flagella (Fig.1) and were actively motile. And the cells of XJU-80 had capsule by the observation of the transmission electron microscope. The XJU-1 isolate grew in nutrient broth at pH between 4.5 and 12.6, and for XJU-80 at pH between 3.8 and 12.8. And the optimum pH of the two isolates is around pH11.5, and with up to 24% and 26% (w/v) NaCl at pH12; thus indicates that they are extremely alkali-tolerant, facultatively acid-tolerant, and halo-tolerant bacteria. The growing temperature range is from 30℃ to 56℃ for XJU-1 and from 30℃ to 58℃ for XJU-80, with an optimum around 39℃ and 42℃, respectively. Interestingly, both strains can grow under the double stress condition of the pH12.5 and 55℃. The XJU-1 and XJU-80 strains were positive for utilization of starch, gelatin, NO_2 from NO_3^-, NO from NO_2^-, citrate, catalase, lecithinase, oxidase and xylan. It was negative for utilization of VP reaction (pH10.5), D-manmitol, L- arabinose, D-xylose, D-fructose, Tween60, Tween40, Tween20, Indole production, phenylalanine deaminase, dextrin and methyl red. And as a rapid screen, there were halos around the inoculation of the two strains on the xylan-medium. So the two isolates could degrade the xylan. According to DNS method, the crude enzymes were found to be active toward xylan at pH12 and stable for 10min in the pH6.5 ~ 12. Both xylanase were stable up to 53℃ and the temperature optimum for activity was about 72℃.

3.2 16S rDNA sequencing and analysis

For further characterization of the two strains XJU-1 and XJU-80, we constructed a phylogenetic tree based on comparison of the 16S rDNA sequence of the isolates and those of type strains of Bacillus species (Fig.2). XJU-1 and XJU-80 formed a tight cluster with Bacillus halodurans. There was 99.9% sequence similarity between XJU-1 and XJU-80; and the sequence relatedness between XJU-1 and Bacillus halodurans C-125 and between XJU-80 and Bacillus halodurans 497^T was high up to 99.8% and 99.9% similarity, respectively. XJU-1 and XJU-80 have very high similarities, but they were divided into different branches. Thus perhaps is the reason that the two strains had already been differentiated before they contributed in Turpan in China.

Table 1　Phenotypic properties of B. halodurans XJU-80 and B. halodurans XJU-1

Characteristics	B. halodurans XJU-80	B. halodurans XJU-1
Gram behavior	+	+
Cell shape	Rods	Rods
Cell size	0.7 – 0.8 × 2.5 – 5.0μm	0.8 – 0.85 × 2.5 – 5.0μm
Spore shape	ellipsoidal	ellipsoidal
Sporangium swollen	+	+
Anaerobic growth	+	+
VP reaction (pH10.5)	–	–
Growth at 40℃	+	+
at 55℃(pH12.5)	+	+
at 58℃	+	–
Growth in medium pH3.8	+	–
medium pH12.6	+	+
medium pH12.8	+	–
Growth in NaCl 0%	–	–
18%	+	+
24%	+	+
26%	+	–
Acid from D-mannitol	–	–
L-arabinose	–	–
D-xylose	–	–
D – fructose	–	–
Hydrolysis of starch	+	+
Gelatin	+	+
Tween 60	–	–
Tween 40	–	–
Tween 20	–	–
NO_2 from NO_3^-	+	+
NO from NO_2^-	+	+
Indole production	–	–
Phenylalanine deaminase	–	–
Use of citrate	+	+
Catalase	+	+
Oxidase	+	+
Lecithinase	+	+
Dextrin	–	–
Cellulose	–	–
Xylan	+	+
Methyl red	–	–

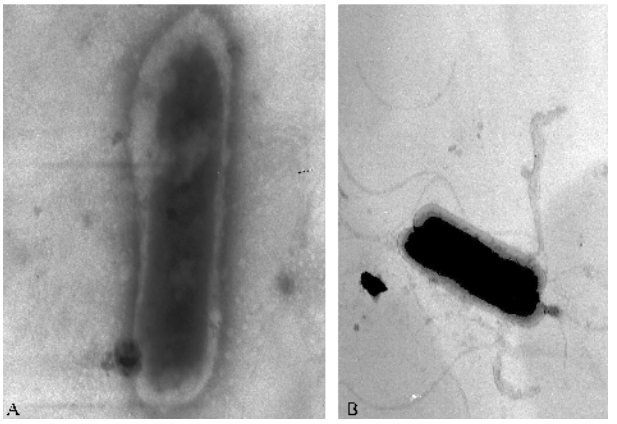

Fig.1　Transmission micrograph of B. halodurans XJU-80(A) and B. halodurans XJU-1(B). The strains were grown aerobically at pH11.5 for 14h. For negative staining, one drop of culture was placed on a copper grid coated with 1% potassium phosphotungstic acid adjusted to pH6.5 with potassium hydroxide. (bar = 1μm).

3.3　DNA-DNA hybridization analysis

The G + C contents of XJU-1 (40.5mol%) and XJU-80 (42.2mol%) are almost identical to that of Bacillus halodurans. DNA-DNA hybridization analysis was carried out comparing the strains XJU-1 and XJU-80 with other standard strains (Table 2). The genomic similarity between strains XJU-1 and XJU-80 was obtained 84% ~ 85% and between our two native strains and B. alcalophilus DSM485 was quitely low (53% and 45%, respectively), thus indicating that strains XJU-1 and XJU-80 was genomically unrelated to B. alcalophilus. While the XJU-1 was closely related with B. halodurans DSM497^T (81.5%) and B. halodurans C-125 (80%); and the XJU-80 was also highly related with B. halodurans DSM497^T (80%) and B. halodurans C-125 (71.5%). The data showed that Bacillus sp. XJU-1 and XJU-80 should be classified as the members of Bacillus halodurans. The hybridization values of other Bacillus type strains used to the strains XJU-1 and XJU-80 were significantly lower (53% and 45%, respectively) than that obtained in the case of B. halodurans as shown in Table 2.

Table 2　DNA-DNA hybridization between Bacillus sp. XJU-1, XJU-80 and other related strains

Strains	G + C mol%	Homology/%				
		B. halodurans XJU-1	B. halodurans XJU-80	B. alcalophilis DSM485	B. halodurans DSM-497^T	B. halodurans C-125
B. halodurans XJU-1	40.5	100	85	53	81.3	80
B. halodurans XJU-80	42.2	84	100	45	80	71.5
Escherichia coli K12	51.7					

T: Type strain.

4　Discussion

Not only on the basis of morphological, physiological and biochemical characteristics but also through phylogenetic analysis, the strains of XJU-1 and XJU-80 had been defined as Bacillus halodurans. We have named the two new strains as Bacillus halodurans XJU-1 and Bacillus halodurans XJU-80.

To date, most isolated Bacillus halodurans strains or subspecies were reported by East Africa[25, 10] and

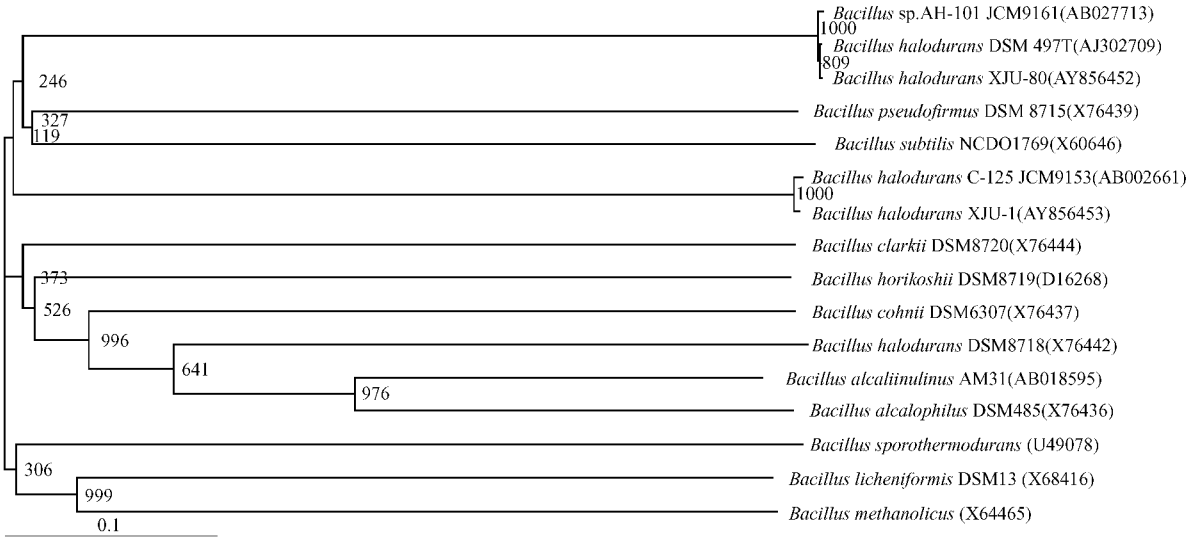

Fig.2 Unrooted Phylogenetic Tree Showing the Relationship of Strains XJU-1 and XJU-80 to other Bacillus Strains. The 16S rDNA sequences of XJU-1 and XJU-80 were related and compared with those of related Bacillus sp. Evolutionary distances were calculated by the neighbour-joining´ method[22]. Numbers at the nodes represent the confidence level from 1000 replicate bootstrap samplings. Bar 0.1 substitutions per nucleotide.

America[6, 26]. Whereas our two isolates were isolated from Xinjiang of the middle area of the Eurasia continent. So the study will provide some clue for the study of the geographical distribution of the strains of Bacillus halodurans species. The result of phylogenetic analysis indicated that the XJU-1 strain was closely related to the Bacillus halodurans C-125, and the XJU-80 strain was tightly clustered with the B. halodurans DSM497^T (Fig. 2). Therefore, it reveals the possibility that the two strains had already been differentiated before they contributed here. The strong tolerance to high alkaline condition of our two isolates (pH12.8 for XJU-80, and pH12.6 for XJU-1) revealed that there would be more alkali-tolerant strains in our planet. And the growing ability of both strains at 55℃ with pH 12.5 condition under double stress, it is expected that in the near future further alkaliphilic and thermophilic micro-organisms will be isolated with grown ranges beyond those presently observed.

The alkaliphiles can grow at pH9 and the value of pH_{opt} is 10 ~ 12, but they can grow weakly or not grow at pH6.5 approximately[27]; while alkali-tolerant bacteria cannot grow at the pH over 10.5, but can grow at pH7 ~ 9[28]. According to the broad range pH phenotype (from 4.5 to 12.6 for XJU-1 and from 3.8 to 12.8 for XJU-80, respectively), we could not illustrate exactly that our two isolates were belonging to alkaliphiles or alkali-tolerant bacteria; but they also seemed to have some facultatively acid-tolerant character. Therefore, the characteristics of our two native isolates suggest that they have adapted various regulatory pathways for their survival under different pH environmental conditions during long stage of evolutionary process. Their adaptation to both high and low pH condition draws our attention not only because they are potential source of industrially valuable enzymes but also because of their adaptive mechanisms to external environmental parameters.

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中国吐鲁番两株产木聚糖酶的极端耐碱 Bacillus halodurans 的分类鉴定

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摘　要:通过生理生化实验、16S rDNA 序列分析和同源性杂交,将分离到的 XJU-1 和 XJU-80 菌种进行了分类鉴定。XJU-1 和 XJU-80 具有较宽的 pH 生长范围(分别是 pH4.5 ~ 12.6 和 pH3.8 ~ 12.6),其 G + C mol% 含量分别是 40.5mol%和 42.2mol%。16S rDNA 序列分析和 DNA-DNA 同源杂交结果表明,XJU-1 和 XJU-80 与 Bacillus halodurans C-125 和 Bacillus halodurans DSM497^T 具有较高的同源性(99%),两者之间也具有 85%的相关性,但其与 Bacillus halodurans C-125 和 Bacillus halodurans DSM497^T 分别具有 81.3%和 71.5%的相关性。基于以上结果,将两株分离菌株分类为 Bacillus halodurans 的两个新品系。

关键词:Bacillus halodurans;G + C mol%含量;16S rRNA;DNA-DNA 同源杂交;进化树分析

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