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A Taxonomic Study of *Bacillus* sp. Isolated from Korean Salt-Fermented Anchovy

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Abstract

A bacterial strain producing an extracellular ß-amylase, designated KYJ 963, was isolated from Korean salt-fermented anchovy (anchovy-*jeot*). A nearly complete nucleotide sequence of a 16S ribosomal RNA from the isolate was determined. On the basis of the results of 16S ribosomal DNA sequence, cellular fatty acid composition, sensitivity test to antibiotics and so on, the strain KYJ 963 was identified as the closest microbe to *Bacillus megaterium* and *Bacillus simplex*.

Introduction

Jeot-gal (or *jeot*) is the traditional fermented fishery products of Korea. More than thirty kinds of *jeot-gal* are processed and consumed in Korea. Various types of fish, shell fish, crustacean, roe, and different NaCl concentrations are used for *jeot-gal* manufacture depending on the types of *jeot-gal* (Lee, 1993; Um and Lee, 1996). *Jeot-gal* is divided broadly into two groups according to the types of fermentation which is determined by the NaCl concentration and the use of added carbohydrate; high- or low-salt *jeot-gal* and low-salt *sikhae*. *Sikhae* is made by blending salted fish with cooked cereals and various seasonings, and allowed to ferment until a suitable acidic taste has formed slightly (primarily lactic acid fermentation) (Lee, 1993). On the other hand, *jeot-gal* is made by mixing fish with NaCl alone. High-salt *jeot-ga*l contains about 25% NaCl of fish weight, whereas low-salt *jeot-gal* is prepared with less than 10% NaCl. Recently, low-salt *jeot-gal* has been actively studied because of the limitation in the consumption of *jeot-gal* by its high-salt concentration (Lee, 1993).

The fermentation of *jeot-gal* is completed by the action of endogenous enzymes as well as microorganisms occurred in the system. Although microorganisms affect primarily the fermentation of *jeot-gal*, microorganisms play an important role in the taste and flavor formation of fermented *jeot-gal*. The microflora in salt-fermented fishery products have been reported by some investigators (Lee, 1993; Um and Lee, 1996; Lee, 1993; Mheen, 1993; Phithakpol, 1993; Tanasupawat *et al*., 1992). In our laboratory, we have isolated *Bacillus, Staphylococcus, Micrococcus, Pseudomonas, Aerococcus,* and *Vibrio* from the fermentation process of anchovy-*jeot*. Several studies have reported the appearance of the genus *Bacillus* in the salt-fermented fishery products (Lee, 1993; Mheen, 1993; Phithakpol, 1993) but did not fully study the taxonomy of the microbes.

Thus, in the present work, we describe the taxonomic characteristics of *Bacillus* sp. KYJ 963 isolated from anchovy-*jeot* in detail and discuss its influences on the *jeot-gal* including *anchovy-jeot*.

Results and Discussion

Identification of the Isolated Strain

Morphological study was examined under a transmission electron microscope. The strain KYJ 963 was a rod-shaped bacterium with peritrichous flagella (data not shown). A nearly complete nucleotide sequence of a 16S ribosomal RNA from the strain KYJ 963 was determined following the isolation and cloning of amplified genes. The 16S rDNA sequence of the strain KYJ 963 has the highest similarity with those of both *B. megaterium* and *B. simplex* (Table 1). The 16S rDNA sequence of the strain KYJ 963 showed 98.7% and 98.2% homology to *B. megaterium* and *B. simplex*, respectively. The 16S rDNA sequence of the strain KYJ 963 was deposited into the GenBank database under accession number AF 217809.

The fatty acid compositions of 19 *Bacillus* species have been studied by Kaneda (Kaneda, 1977), who divided these organisms into six groups (Kaneda groups A-F). All groups except D, in which cyclohexane fatty acids are most abundant, contain numerous branched chain acids (iso and anteiso). Table 2 shows characteristic profiles of the cellular fatty acid composition of the strain KYJ 963. The major cellular fatty acids found in this strain were iso-$C_{15:0}$ (36.88%) and anteiso-$C_{15:0}$ (28.14%), and small proportions of unsaturated fatty acids were also found. The range of chain length was 13-19. Thus, the strain KYJ 963 belongs to Kaneda group A (*B. megaterium, B. subtilis, B. alvei, B. brevis, B. circulans, B. licheniformis, B. pumilus,* and *B. macerans*) in which anteiso-$C_{15:0}$ (26-60%) and iso-$C_{15:0}$ (13-30%) occur as the most abundant fatty acid. On the basis of the 16S ribosomal DNA sequence and cellular fatty acids analysis, the strain KYJ 963 was classified as a species of the genus *Bacillus*.

Sensitivity Test to Antibiotics

There are few studies of the sensitivity of *Bacillus* species to antibiotics even though they relate to the genus taxonomy. *Bacillus* sp. KYJ 963 showed resistance to the antibiotics penicillin G. Whereas, it showed sensitivity to cephalosporin C, ampicillin, polymyxin B, bacitracin, kanamycin, rifampicin, tetracycline, streptomycin,

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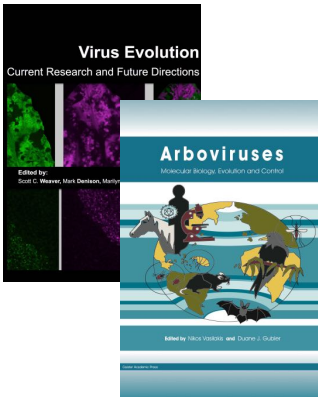

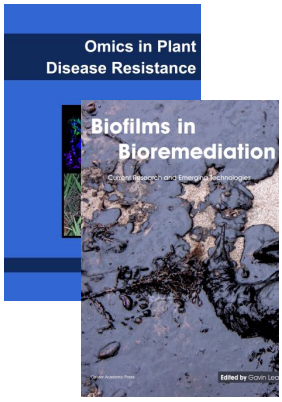

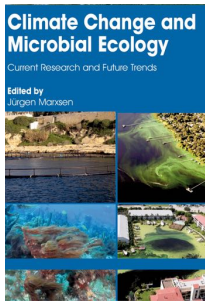

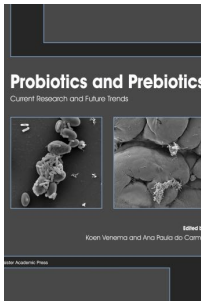

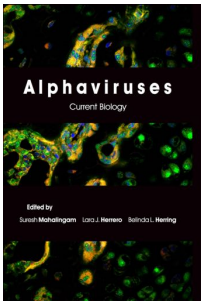

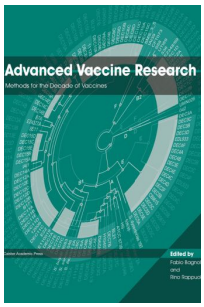

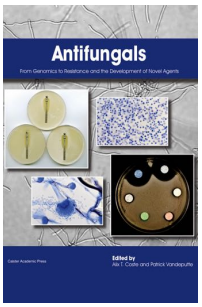

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Table 1. Matrix of 16S rDNA sequence similarity values (%) between strain KYJ 963 and other closely related *Bacillus* species.

Species	1	2	3	4	5	6	7	8	9	10	11		
1 Strain KYJ 963													
2 *Bacillus megaterium* IAM 13418^T	98.7												
3 *Bacillus simplex* DSM 1321^T	98.2	98.9											
4 *Bacillus firmus* IAM 12464^T	96.2	95.5	95.5										
5 *Bacillus cohnii* DSM 6307^T	96.1	95.7	95.9	94.7									
6 *Bacillus circulans* IAM 12462^T	96.0	96.0	95.5	96.4	95.3								
7 *Bacillus halmapalus* DSM 8723^T	95.8	95.5	95.2	94.5	97.7	94.6							
8 *Bacillus cereus* IAM 12605^T	95.4	94.7	94.5	94.2	95.1	93.9	95.6						
9 *Bacillus methanolicus* NCIMB 13113^T	95.3	95.0	95.1	96.3	94.8	95.3	94.5	93.5					
10 *Bacillus licheniformis* DSM 13^T	94.1	93.5	94.0	95.4	94.3	93.8	94.4	93.9	95.0				
11 *Bacillus subtilis* NCDO 1769^T	94.0	93.9	93.9	94.9	94.0	93.7	93.9	94.0	95.4	98.2			
12 *Bacillus coagulans* JCM 2257^T	92.7	92.7	92.4	93.5	92.6	92.3	92.1	92.7	94.1	92.9	94.0		

chloramphenicol, and erythromycin (Table 3). Interestingly, the sensitivity patterns to antibiotics among *Bacillus* sp. KYJ 963, *B. megaterium*, and *B. simplex* were very similar.

Growth Characteristics and Extracellular Products of *Bacillus* sp. KYJ 963

Salt tolerance, growth temperature range, growth pH range, and extracellular products are important taxonomic criteria which are used to differentiate species in the genus *Bacillus* (Claus and Berkeley, 1986). *Bacillus* sp. KYJ 963 grew in media with 0 to 15% NaCl with an optimal growth of 0% NaCl (data not shown). In a medium with 15% NaCl, *Bacillus* sp. KYJ 963 started to grow after 58 hr from its inoculation, but did not grow at all for one month in a medium with 16% NaCl. It also grew at 6.0 to 10.0 with an optimal pH of 7.5, and at 15 to 50°C with an optimal growth temperature of 37°C (data not shown). *Bacillus* sp. KYJ 963 excreted an extracellular amylase in large quantities into the growth medium and the amylase could be renatured and detected *in situ* after SDS-gel electrophoresis (Figure 1). However, the activity of protease or esterase was only slightly detected on a protease indicate agar plate (Kim *et al.*, 1998) or an esterase indicate agar plate (Kok *et al.*, 1993). In the previous study, we purified an extracellular amylase easily by using starch adsorption technique from *Bacillus* sp. KYJ 963 and reported its enzymatic properties (Ha *et al.*, 2001). That is, the molecular weight of the purified enzyme was approximately 59, 000 and the enzyme was a novel ß-amylase which could not hydrolyze maltose or α-cyclodextrin.

Effect of NaCl Concentration on the Activity of the Extracellular ß-Amylase

We examined the effect of NaCl on the extracellular ß-amylase activity because *Bacillus* sp. KYJ 963 was isolated from high-salt anchovy-*jeot*. Figure 2 shows that the activiy of the ß-amylase is inactivated to approximately 16% and

Table 2. Cellular fatty acid composition of the strain KYJ 963 analysed by gas-liquid chromatography.

Fatty acids	Retention time	Percentage
13:0 IS O	5.292	0.36
14:0 ISO	6.331	3.87
14:0	7.060	2.74
15:0 ISO	8.003	36.88
15:0 ANTEISO	8.139	28.14
16:1 ω7c alcohol	9.205	1.94
16:0 ISO	9.579	1.66
16:1 ω11c	15.757	7.82
16:0	10.207	3.33
ISO 17:1 ω10c	10.870	3.13
17:0 ISO	11.284	3.38
17:0 ANTEISO	11.442	3.96
18:1 ω9c	13.251	0.32
18:0	13.660	0.39
19:0 ISO	14.780	0.17

50% of the control (0.0% NaCl) at 4% and 15% NaCl concentrations, respectively.

It is known that the NaCl concentration and pH of salt-fermented fishery products (*jeot-gal*) are about 10-25% and 4.8-7.1, respectively (Um and Lee, 1996). Also, the final pH and NaCl concentration of Korean salt-fermented anchovy (anchovy-*jeot*) were about 5.8 and 25%, respectively (our experimental results). *Bacillus* sp. KYJ 963 can grow at the pHs of 6.0 and over, and the NaCl concentrations of 15% and below, and the activities of its extracellular ß-amylase at pH 6.0 and at 15% NaCl concentration exhibited about 66% of pH 7.5 and 50% of 0.0% NaCl, respectively (Ha *et al.*, 2001; Figure 2). Thus, on the basis of the results concerning the physiological

Table 3. Sensitivity patterns of the several *Bacillus* species to various antibiotics.

Antibiotics	*Bacillus sp.* KYJ 963	*Bacillus megaterium* KCTC 1098	*Bacillus simplex* KCTC 3450	*Bacillus cereus* KCTC 3674	*Bacillus subtilis* ATCC 10774
Penicillin G	+	+	+	+	+
Cephalosporin C	−	−	−	+	−
Ampicillin	−	−	−	+	+
Polymyxin B	−	−	−	+	−
Bacitracin	−	−	−	+	+
Kanamycin	−	−	−	−	−
Rifampicin	−	−	−	−	−
Tetracycline	−	−	−	−	−
Streptomycin	−	−	−	−	−
Chloramphenicol	−	−	−	−	−
Erythromycin	−	−	−	−	−

Symbol +: resistant, −: sensitive

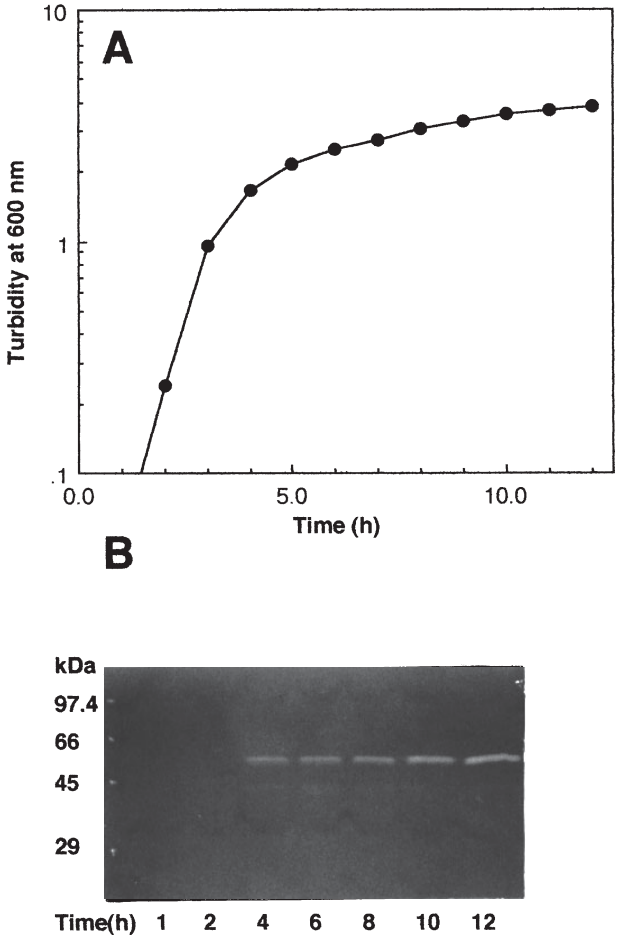

Figure. 1 Growth curve and time course of extracellular amylase production by *Bacillus* sp. KYJ 963.
(A) Cell density was measured by absorbance at 600 nm (closed circles) Samples (1 ml) were withdrawn at times from the culture, centrifuged at 12000 x g for 20 min at 4°C, and the supernatants were retained for SDS-PAGE. (B) Amylolytic activity was detected *in situ* after SDS-PAGE, as described in materials and methods. A 12.5% gel was used to analyze 15 μl of culture supernatant.

characteristics and extracellular ß-amylase of *Bacillus* sp. KYJ 963, it is believed that *Bacillus* sp. KYJ 963 can affect a sweet taste of *jeot-gals* including s*ikhae* during the fermentation process at the NaCl concentrations of 15% and below, and the pHs of 6.0 and over.

Experimental Procedures

Isolation of the Extracellular Amylase-Producing Bacterial Strain

During the fermentation of anchovy-*jeot*, samples of anchovy-*jeot* were plated on an isolation medium composed of 0.5% polypeptone, 0.5% yeast extract, 0.2% KCl, 0.2% $MgSO_4$, 0.001% $FeCl_2$, 5% NaCl, 1% soluble starch, and 1.5% agar. A bacterial strain that formed a zone of clearance around the colonies with a solution of 10 mM I_2-KI was isolated and purified.

Bacterial Strains and Growth Conditions

The bacterial strains used in this study were KYJ 963 isolated from anchovy-*jeot*, *Bacillus megaterium* KCTC 1098, *Bacillus simplex* KCTC 3450, *Bacillus cereus* KCTC 3674, and *Bacillus subtilis* ATCC 10774. All bacteria were aerobically cultured at 37°C. Isolate KYJ 963 was grown in a liquid medium containing 0.5% polypeptone, 0.5% yeast extract in 50 mM Tris-HCl (pH 7.5).

Media for Sensitivity Test to Antibiotics

Antibiotics were included in the agar medium containing 0.5% polypeptone, 0.5% yeast extract, and 1.5% agar at the following concentrations: 50 units/ml penicillin G, 100 μg/ml cephalosporin C, 50 μg/ml ampicillin, 1530 units/ml polymyxin B, 7.1 units/ml bacitracin, 50 μg/ml kanamycin, 30 μg/ml rifampicin, 50 μg/ml tetracycline, 50 μg/ml streptomycin, 50 μg/ml chloramphenicol, and 40 μg/ml erythromycin.

Cellular Fatty Acid Analysis and 16S Ribosomal DNA Sequencing of the Strain KYJ 963

Cellular fatty acids were extracted and analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID, Newark, USA). The 16S rDNA was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). The PCR product was purified by using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA, USA). The purified 16S rDNA was sequenced using ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit (Applied Biosystems) as recommended by the manufacturer. The purified sequencing reaction mixtures were automatically electrophoresed using an Applied Biosystems model 310 automatic DNA sequencer. The 16S rDNA sequence of the strain KJY 963 was aligned with 16S rRNA gene

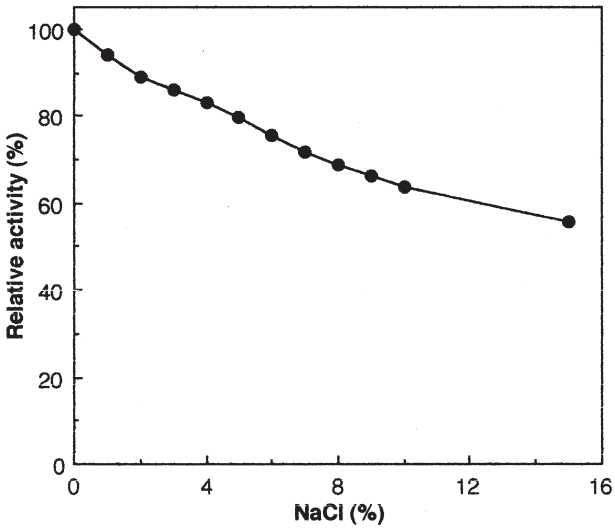

Figure. 2 Effect of NaCl concentration on the activity of purified ß-amylase.

sequences of *Bacillus* species and the representatives of some related genera by using CLUSTAL W software (Thompson *et al*, 1994). Gaps at the 5'and 3' ends of the alignment were omitted from further analyses. Evolutionary distance matrices were calculated by using the algorithm of Jukes and Cantor (Jukes and Cantor, 1969) with the DNADIST program within the PHYLIP package (Felsenstein, 1993).

Detection of Enzyme Activity on PAGE

An extracellular amylase produced by strain KYJ 963 was detected on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.2% (w/v) starch as a copolymerized substrate. SDS-PAGE process was completed with a 12.5% gel by following the Laemmli method (Laemmli, 1970). Samples were heated at 100°C for 2 min before they were put on. After PAGE the gel was washed in Triton X-100 (2.5%, v/v) for one hour at room temperature to remove the SDS and restore enzyme activity, then incubated in 0.1 M glycine buffer (pH 7.5) containing 1 mM $MnCl_2$ and 1 mM $FeCl_2$ for three hours at 45°C. The gel was treated with a solution of 10 mM I_2-KI to stop the reaction and stain the unreacted starchbackground. The zones of amylase activity appeared as light bands against a dark background.

Enzyme Assay

Measurement of extracellular amylase activity was followed by the dinitrosalicylic acid procedure (Kim *et al*, 1999). Hydrolysis of 0.5 ml of soluble starch (1%, w/v, in 50 mM Tris-HCl, pH 7.5) was carried out in a mixture containing 0.1 ml of enzyme sample and 0.4 ml of 50 mM Tris-HCl (pH 7.5). After incubation for 30 min at 45°C the reaction was stopped by the addition of 1 ml of 3,5-dinitrosalicylic acid reagent. The samples,
along with a reagent blank, were heated in a boiling-water bath for 7 min, and immersed in an ice bath. After five-fold dilution with distilled water, the absorbance was read at 540 nm.

Acknowledgements

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