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Taxonomic Studies of the Beta Hemolysis-causing Pathogen *Bacillus cereus* Isolated from Sea Water

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Abstract A bacterial strain that excretes hemolysins and proteases into the growth medium was isolated from sea water and designated as KYJ 961. A nearly complete nucleotide sequence of a 16S ribosomal RNA gene from the isolate was determined following the isolation and cloning of amplified genes. On the basis of the 16S ribosomal DNA sequence data, and morphological, chemotaxonomic, and physiological characteristics, strain KYJ 961 was classified as a strain of *Bacillus cereus*.

Key words: *Bacillus cereus*, taxonomic characteristics, 16S rRNA gene sequence

The *Bacillus cereus* group, including *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis*, is medically and industrially important. However, taxonomic classification of species within this group is so controversial because of many similarities between taxa, and several microbiologists have questioned the taxonomic position of *B. cereus* group members as separate species [9, 25]. As an example of this similarity, DNA-DNA hybridization studies on the *B. cereus* group have shown that the species within the group share chromosomal base sequence similarities [18, 24, 26], and an rRNA sequence comparison between *B. cereus* and *B. anthracis* has revealed an identical 16S rRNA nucleotide sequence and only two differences in the 23S rRNA sequence [2, 3]. Thus, rRNA sequence and DNA-DNA hybridization data from the *B. cereus* group appear to be insufficient to ensure species identity. However, the *B. cereus* group members can be distinguished phenotypically. *B. cereus* is a gram-positive facultative anaerobic spore-forming rod-shaped bacterium. This bacterium is responsible for an increasing number of food poisoning cases which are characterized by diarrhea and emesis [15, 20]. A number of studies of *B. cereus* have documented its extracellular products [1, 4, 16, 27, 29]. These products include hemolysins, diarrheogenic enterotoxin, emetic toxin, neutral (metallo-) protease, phospholipase C, and Rho-ADP-ribosyltransferase. *B. anthracis* is the causative agent of anthrax in both humans and animals. Virulent isolates of the organism carry two plasmids, pXO1 [21] and pXO2 [10, 28], both of which are required to produce the disease. The three toxin genes are located on a large (114 megadaltons) plasmid, pXO1, and the capsule gene is located on a smaller (60 kilodaltons) plasmid, pXO2. Avirulent pXO1- and/or pXO2-deficient strains are virtually indistinguishable from both *B. cereus* and *B. thuringiensis*. *B. thuringiensis* is a well known insect pathogen, and is the source of the most widely used biological pesticide in the world today. This gram-positive soil bacterium differs from both *B. cereus* and *B. anthracis* in producing a crystal protein toxin during sporulation that is pathogenic to various insects, notably lepidoptera, diptera, and coleoptera [14]. Plasmids of *B. thuringiensis* coding for insecticidal crystals have been transferred to *B. cereus* [8]. Such *B. cereus* transconjugants can be considered to be changed to *B. thuringiensis* since these organisms synthesize crystal proteins. Thus, the *B. cereus* group is of taxonomic interest.

Morphology, salt tolerance, growth temperature range, extracellular products, utilization pattern of carbon sources, isoprenoid quinone system, and antibiotic sensitivity are important taxonomic criteria which are used to differentiate species in the genus *Bacillus* [5]. However, not enough is known about these taxonomic criteria to distinguish the *B. cereus* strain from other members of *B. cereus* group.

B. cereus KYJ 961 is the first *B. cereus* strain to be isolated from sea water, and also the first one to be

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found and precisely identified in Korea. In the present work, the identification of the beta hemolysis-causing pathogen *B. cereus* KYJ 961 is described, and is related to public health in a seaside resort.

MATERIALS AND METHODS

Isolation of the Extracellular Protease-producing and Hemolysin-producing Bacterial Strain

Sea water samples obtained from the seaside resort of Masan, Korea were collected and plated on an isolation medium composed of 1% skim milk, 0.5% polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4, 0.2% glucose, 3% NaCl, and 1.5% agar. A bacterial strain that formed a clear zone of proteolysis around colonies was isolated and purified by streaking on isolation medium plates. The strain was also streaked onto blood agar supplemented with 5% defibrinated goat blood, 50% beef heart (infusion form), 1% tryptose, 0.5% NaCl, and 1.5% agar to examine hemolytic activity.

Extracellular Protease Assay

A preculture grown overnight was used to inoculate the main culture to give a turbidity of approximately 0.5. Aliquots (1 ml) were withdrawn from the main culture, then centrifuged at 15000 rpm for 20 min at 4°C. The supernatant was retained for analysis. Measurement of extracellular protease activity followed the procedure of Prestige *et al.* [23] with minor modifications. Hydrolysis of 0.25 ml of azocasein (2% in water) was carried out in a mixture containing 0.15 ml of enzyme sample, 50 µl of 1 M MES-NaOH (pH 6.5), and 50 µl of distilled water. After incubation for 30 min at 45°C the reaction was stopped by the addition of 1 ml of 7% perchloric acid. The mixture was centrifuged at 15,000 rpm for 10 min at 4°C. After addition of 0.15 ml of 10 N NaOH to 1 ml of the supernatant, the absorbance was measured at 436 nm.

Identification of the Isolated Strain

The flagellation and the morphology of vegetative cells were examined under both a transmission and a scanning electron microscope. Capsule determination in the isolated strain was done using the Hiss staining method [13]. For total cellular fatty acid analysis, bacterial cells were harvested from Trypticase soy agar. Fatty acids were extracted by following the Microbial Identification System (MIDI; Microbial ID, Inc., Newark, DE, U.S.A.) instructions as described by Yang *et al.* [30]. For analysis of the 16S rDNA sequence, 16S rDNAs were amplified with the two primers 5'-GAGTTTGATCCTG GCTCAG-3' (in *Escherichia coli*, 16S rRNA from positions 9 to 27), and 5'-AGAAAGGAGGTGATCCAGCC-3' (in *E. coli*, 16S rRNA from positions 1542 to 1525). Cloning and sequencing of amplified 16S rDNA were done using the method of Kim *et al.* [19]. The Biolog System (Biolog, Inc., Hayward, CA, U.S.A.), which is an automated identification and classification system for microorganisms, was used to examine the carbon source utilization pattern with regard to 95 different carbon sources, including amino acids, carboxylic acids, and carbohydrates. The isolated strain was subcultured onto BUGM (Biolog Universal Growth Medium, Biolog, Inc. Hayward, CA, U.S.A., # 70001) agar and incubated at 30°C for 24 h. Cells were scraped and suspended in 0.85% NaCl solution according to the manual of the Biolog System.

Bacterial Strain and Growth Conditions

The bacterial strain used in this study was KYJ 961 isolated from sea water. It was grown aerobically at 37°C. For extracellular protease assay, the strain was grown in a liquid medium containing 0.5% polypeptone, 0.5% yeast extract, and 0.5% NaCl in 50 mM phosphate buffer (pH 6.5). For sensitivity test to antibiotics, the strain was grown on agar plates containing 0.5% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 0.5% agar. The antibiotics penicillin G (50 units), cephalosporin C (100 µg/ml), ampicillin (60 µg/ml), tetracycline (50 µg/ml), streptomycin (50 µg/ml), kanamycin (50 µg/ml), rifampicin (40 µg/ml), chloramphenicol (50 µg/ml), bacitracin (14 units), and polymyxin (1530 units) were added as needed.

RESULTS

Extracellular Products of Strain KYJ 961

Strain KYJ 961 produced extracellular proteases. Secretion of extracellular proteases occurred during the logarithmic growth phase and was greatest when cultures reached the stationary growth phase (Fig. 1A). As shown in Table 1, the proteolytic activity of culture supernatant was stimulated by Ca^{2+} and Zn^{2+}, and inhibited strongly by either a metal chelator, EDTA (4 mM), or a zinc-specific chelator, 1,10-phenanthroline (0.5 mM). However, the proteolytic activity was resistant to phenylmethylsulfonyl fluoride (PMSF) and iodoacetic acid. The optimum pH of extracellular proteases was 6.5 (Fig. 1B). These results indicate that the main proteases in the culture supernatant of strain KYJ 961 are neutral (metallo-) proteases. Strain KYJ 961 also showed beta hemolysis surrounding colonies on 5% goat blood agar, indicating that its hemolytic activity was due to the secretion of hemolysins (Fig. 1C). The activities of amylases, esterases, lipases, and chitosanases were not detected in the culture supernatant of strain KYJ 961.

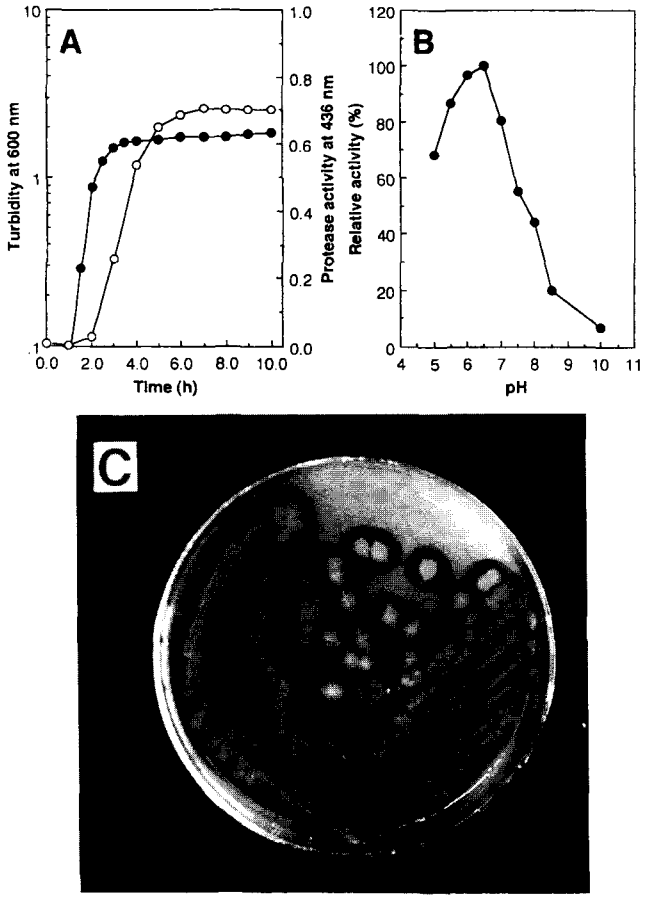

Fig. 1. Extracellular proteolytic and hemolytic activities of strain KYJ 961.

(A) Extracellular proteolytic activity. Cell density was measured by absorbance at 600 nm (closed circles) and extracellular proteolytic activity was measured by absorbance at 436 nm (open circles). (B) Effect of pH on the extracellular proteolytic activity. The proteolytic activity of culture supernatant was determined with different ranges of pH. Buffers used at a concentration of 100 mM were MES (pH 6 to 6.5), HEPES (pH 7 to 8), Tricine (pH 8 to 8.5), and CAPSO (pH 10). (C) Extracellular hemolytic activity.

Cellular Fatty Acid Composition and 16S Ribosomal RNA Gene Sequence of Strain KYJ 961

The fatty acid composition of 19 *Bacillus* species were investigated by Kaneda [17] 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 strain KYJ 961. The predominant fatty acids of this strain were *iso*-C15 : 0 (31.31%), *iso*-C17 : 0 (10.26%), *iso*-C13 : 0 (7.85%), *iso*-C16 : 0 (6.79%), *anteiso*-C15 : 0 (5.72%), and small proportions (0.3-4%) of unsaturated fatty acids. Thus, strain KYJ 961 belongs to Kaneda group E (*B. cereus* group) in which *iso*-C15 : 0 acid (19~31%) occurs as the most abundant fatty acid.

Table 1. Effects of protease inhibitors and mineral salts on the activity of extracellular proteases from strain KYJ 961.

Compounds	Concentration (mM)	Relative activity (%)
None		100
EDTA	1	10
	4	2
Phenanthroline	0.1	91
	0.5	2
PMSF	1	70
	4	60
Iodoacetic acid	1	98
	4	97
$CaCl_2$	1	130
	4	140
$MgCl_2$	1	100
	4	99
$MnCl_2$	1	100
	4	52
$FeCl_2$	1	79
	4	10
$NiCl_2$	1	14
	4	0
$CuCl_2$	1	11
	4	0
$ZnCl_2$	0.07	126
	1	74
$HgCl_2$	1	29
	4	11

Table 2. Cellular fatty acid composition of strain KYJ 961 analyzed by gas chromatography.

Fatty acids	Retention time	Percentage
12 : 0 ISO	3.95	0.54
13 : 0 ISO	4.935	7.85
13 : 0 ANTEISO	5.031	1.19
14 : 0 ISO	6.146	4.57
14 : 0	6.639	2.66
15 : 0 ISO	7.553	31.31
15 : 0 ANTEISO	7.684	5.72
15 : 0	8.109	0.44
16 : 1 ω7c alcohol	8.725	0.72
16 : 0 ISO	9.109	6.79
16 : 1 ω11c	9.319	0.32
16 : 0	9.709	5.51
15 : 0 2OH	10.07	0.45
ISO 17 : 1 ω10c	10.359	2.20
ISO 17 : 1 ω5c	10.482	3.95
17 : 1 ANTEISO A	10.617	1.02
17 : 0 ISO	10.767	10.26
17 : 0 ANTEISO	10.922	2.04
17 : 1 ω5c	11.252	0.55
18 : 0	13.11	0.42

As shown in Table 3, a comparison of 16S rDNA sequences between strain KYJ 961 and other closely related *Bacillus* species shows that the 16S rDNA

Table 3. Matrix of 16S rDNA sequence similarity values (%) between strain KYJ 961 and other closely related *Bacillus* species.

Species	*Sporolacto-bacillus dextrus* IAM 12380	*Bacillus cereus* IAM 12605	*Bacillus thuringiensis* IAM 12077	*Bacillus licheniformis* DSM 13	*Bacillus anthracis*	*Bacillus subtilis* IAM 12118	Strain KYJ 961
Sporolactobacillus dextrus IAM12380							
Bacillus cereus IAM12605	95.0						
Bacillus thuringiensis IAM12077	94.8	99.7					
Bacillus licheniformis DSM13	93.5	93.7	93.7				
Bacillus anthracis	94.8	99.9	99.6	93.7			
Bacillus subtilis IAM 12118	92.9	93.8	93.8	98.2	94.0		
Strain KYJ 961	95.0	99.9	99.6	93.8	99.9	93.8	

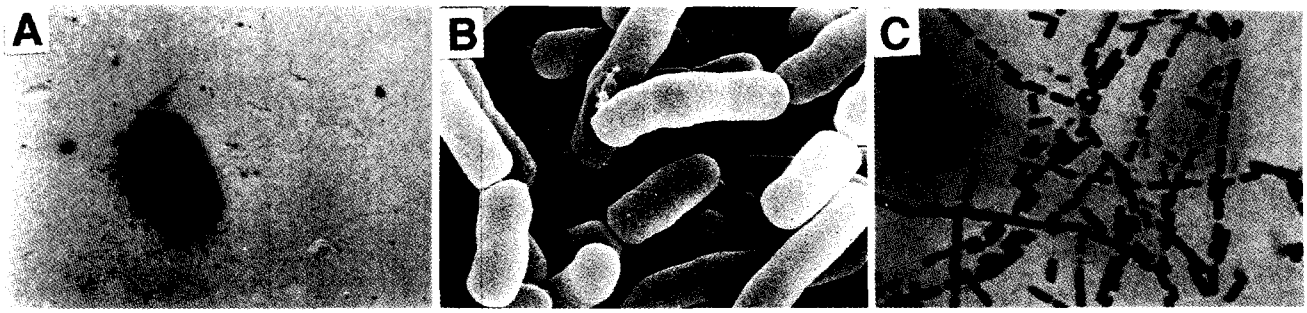

Fig. 2. Morphology of strain KYJ 961.
(A) Transmission electron micrograph of strain KYJ 961 showing peritrichous flagella. (B) Scanning electron micrograph of strain KYJ 961 showing shape and size. A single cell is approximately 3 μm long. (C) Demonstration of the absence of a capsule in strain KYJ 961 by the Hiss staining method observed under phase contrast microscopy.

sequence of strain KYJ 961 was almost identical with sequences of both *B. cereus* and *B. anthracis*. The 16S rDNA sequence of strain KYJ 961 (*Bacillus cereus* KYJ 961) was entered into the Gen Bank database under accession number AF 027659. Thus, additional taxonomic studies are required to determine whether strain KYJ 961 is actually either *B. cereus* or *B. anthracis*.

Cell Morphology and Flagellation

Strain KYJ 961 is a gram-positive facultative anaerobic spore-forming rod-shaped bacteria. Figure 2 shows that strain KYJ 961 has a noncapsulated rod-shape with peritrichous flagella. This single cell is approximately 3 μm in length and 1 μm in width. *B. anthracis* is normally nonmotile, whereas *B. cereus* is normally motile. *B. anthracis* grows in longer chains than both *B. cereus* and *B. thuringiensis*. The latter two species are easily distinguished from virulent strains of *B. anthracis* by virtue of the fact that all virulent strains produce capsules. Based on extracellular products, cellular fatty acid composition, the 16S rDNA sequence, and morphological characteristics, strain KYJ 961 was identified as a strain of *B. cereus*.

Utilization of Carbon Sources

In order to obtain more taxonomic information, we studied *B. cereus* KYJ 961 using Biolog's automated identification system for its ability to use carbon sources. The strain could use β-cyclodextrin, dextrin, glycogen, cellobiose, D-fructose, α-D-glucose, maltose, maltotriose, D-trehalose, D-xylose, D-lactic acid methyl ester, L-lactic acid, L-malic acid, methyl pyruvate, pyruvic acid, N-acetyl L-glutamic acid, L-alanyl-glycine, adenosine, 2'-deoxy adenosine, inosine, thymidine, uridine, adenosine-5'-monophosphate, thymidine-5'-monophosphate, uridine-5'-monophosphate, and D-L-α-glycerol phosphate.

Isoprenoid Quinone System

Menaquinones are the only isoprenoid quinones found in all *Bacillus* strains examined. Menaquinones with seven isoprene units (MK-7) are the main quinone type found in the genus *Bacillus*, and minor components include

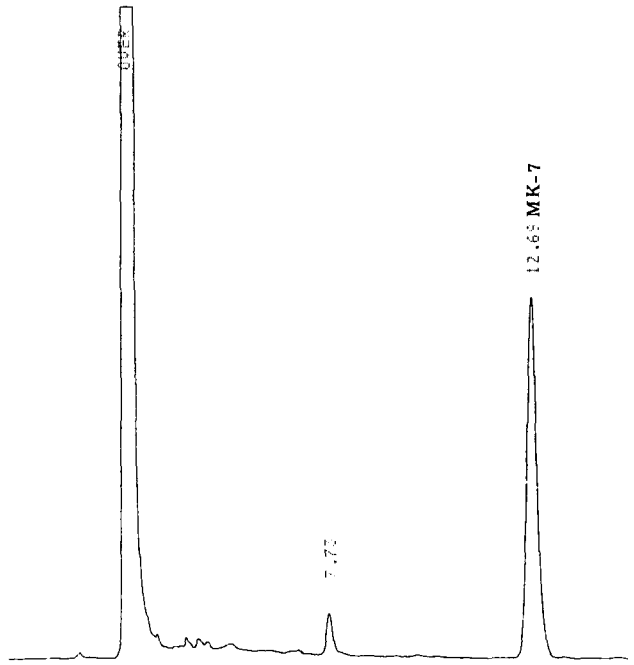

Fig. 3. HPLC chromatogram of menaquinone-7 from *B. cereus* KYJ 961.

MK-2 to MK-11, depending on the species [6]. Several *Bacillus* species possess MK-8 or MK-9 as the major isoprenoid quinone, and a strain of *B. thuringiensis* possesses MK-8 in addition to MK-7 as the major isoprenoid quinone [11]. The respiratory quinone system of *B. cereus* KYJ 961 was examined by the method of Collins *et al.* [7, 12]. *B. cereus* KYJ 961 contained mainly menaquinone with seven isoprene units (93%) as the respiratory quinone (Fig. 3).

Growth Characteristics

Salt tolerance and growth temperature range are important taxonomic criteria which are used to differentiate species in the genus *Bacillus* [5]. *B. cereus* KYJ 961 grew in media with 0 to 10% NaCl with optimum growth at 0% NaCl (Fig. 4A). In a medium with 10% NaCl, *B. cereus* KYJ 961 started to grow after 3 days (data not shown). It grew in temperatures ranging from 15 to 45°C, but did not grow at 10°C and below, or at 50°C and above (Fig. 4B). Thus, *B. cereus* KYJ 961 is a mesophilic, halotolerant bacterium. In a tyrosine decomposition test *B. cereus* KYJ 961exhibited the clearing of tyrosine crystals around colonies on 0.5% tyrosine agar plate.

Sensitivity Test to Antibiotics

There are few studies of the sensitivity of *Bacillus* species to antibiotics even though they relate to the genus taxonomy. As shown in Table 4, *B. cereus* KYJ 961 showed resistance to the antibiotics penicillin G, cephalosporin C,

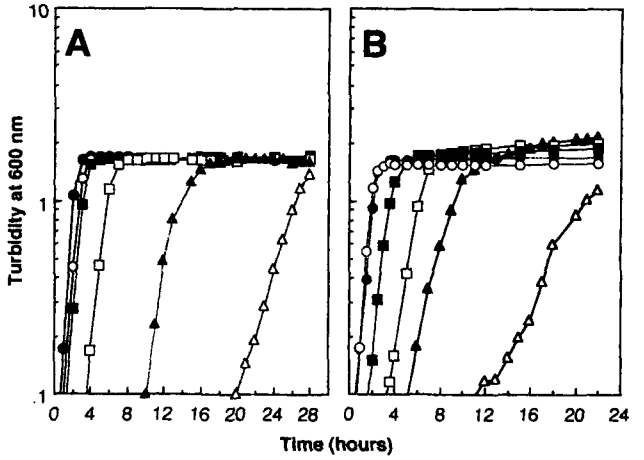

Fig. 4. Effects of NaCl concentration and temperature on the growth of *B. cereus* KYJ 961.

(A) The concentrations of added NaCl were: 0% (closed circles), 2% (open circles), 4% (closed squares), 6% (open squares), 8% (closed triangles), 9% (open triangles). (B) The growth of *B. cereus* KYJ 961 was determined by measuring the cell density at 600 nm for cultures grown at 15° (open triangles), 20° (closed triangles), 25° (open squares), 30° (closed squares), 37° (closed circles), and 45°C (open circles) in liquid media.

Table 4. Sensitivity of *B. cereus* KYJ 961 to various antibiotics.

Antibiotics	*Bacillus cereus* KYJ 961
Penicillin G	–
Cephalosporin C	–
Ampicillin	–
Polymyxin	–
Kanamycin	+
Rifampicin	+
Tetracycline	+
Streptomycin	+
Chloramphenicol	+
Bacitracin	+

Symbol –, resistant; +, sensitive.

ampicillin, and polymyxin, whereas it showed sensitivity to tetracycline, streptomycin, kanamycin, rifampicin, chloramphenicol, and bacitracin. Resistance to the β-lactam antibiotics penicillin G and cephalosporin C is probably due to the activity of β-lactamase.

DISCUSSION

The three species *B. cereus*, *B. anthracis*, and *B. thuringiensis* are closely related since molecular systematic results, including rRNA gene sequences and G+C contents, are similar. The primary criteria used to classify a bacterium as *B. cereus*, *B. anthracis*, or as *B. thuringiensis*, are mainly dependent on what kinds of toxins are produced. However, the occurrence of mutants

and transconjugants among these species makes it difficult to differentiate species within *B. cereus* group. Thus, we need to gain a better systematic understanding concerning the taxonomic criteria in order to distinguish species within *B. cereus* group. However, there are some phenotypic tests, such as sensitivity to penicillin G (*B. cereus* strains show a high resistance to penicillin G due to the activity of a chromosomal β-lactamase; *B. anthracis* strains are generally sensitive to penicillin G, but one resistant strain is now known.), hemolysin activity (*B. cereus* is positive, and *B. anthracis* is negative.), motility (*B. anthracis* is normally nonmotile but one motile strain has been identified; *B. cereus* is normally motile but nonmotile strains are sometimes encountered.), and tyrosine decomposition (*B. anthracis* is negative and *B. cereus* is positive.), which provide useful taxonomic information.

The results of this study, including extracellular products, utilization pattern of carbon sources, salt tolerance, growth temperature range, Isoprenoid quinone system, and antibiotic sensitivity should contribute to differentiation of the *B. cereus* strain from other members of the *B. cereus* group.

B. cereus is known to cause foodborne illness. *B. cereus* is also considered one of the most destructive organisms to affect the eye [22]. Endophthalmitis, which is caused by this organism, is characterized by severe pain, chemosis, periorbital swelling, and extreme proptosis. Late symptoms include corneal edema and corneal ring abscess. *B. cereus* KYJ 961 was isolated from seawater near Masan, so public health should be an important issue. *B. cereus* KYJ 961 grew in media with 0 to 10% NaCl with optimum growth at 0% NaCl (Fig. 4A) between 15~45°C (Fig. 4B). Considering that the NaCl concentration of sea water is approximately 3.5%, *B. cereus* KYJ 961 can grow well in sea water during the summer. Thus, precautions should be taken to prevent *B. cereus* related disease in the summer.

B. cereus KYJ 961 exhibited sensitivity to tetracycline, streptomycin, kanamycin, rifampicin, chloramphenicol, and bacitracin (Table 4). These results should be applied to the medical treatment of *B. cereus* caused disease.

Acknowledgments

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