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Identification of Alkaliphilic *Bacillus* Species Isolated from Lake Van and Its Surroundings by Computerized Analysis of Extracellular Protein Profiles

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Abstract: Five reference *Bacillus* species and 17 native alkaliphilic *Bacillus* strains, isolated from the water of Lake Van and the soil surrounding it, were identified using phenotypic characteristics and extracellular protein profiles by the method of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). According to the phenotypic characteristics, all the native isolates were Gram-positive, aerobic, endospore-forming, motile, facultatively alkaliphilic *Bacillus* spp. It was also found that the native alkaliphilic *Bacillus* isolates were distinguished phenotypically from other *Bacillus* species.

A numerical analysis based on the resulting extracellular protein profiles revealed 5 distinct clusters: native isolates and *B. megaterium* formed 4 clusters that varied between 69% and 100% similarity, and the other reference *Bacillus* species formed a cluster with a similarity of above 70%. The research showed that extracellular protein profiles obtained by SDS-PAGE provide an effective approach to the investigation of taxonomic relationships within native alkaliphilic *Bacillus* isolates and other *Bacillus* species.

Key Words: Lake Van, identification, facultatively alkaliphilic *Bacillus* spp., phenotypic characterization, SDS-PAGE

Van Gölü ve Çevresinden İzole Edilen Alkalofilik *Bacillus* Türlerinin Ekstraselülar Protein Profillerinin Bilgisayar Analiziyle İdentifikasyonu

Özet: Bu çalışmada, 5 referans *Bacillus* türü ve Van Gölü suyu ve çevresindeki topraklardan izole edilen toplam 17 yerel alkalofilik *Bacillus* izolatı fenotipik özellikleri ve sodyum dodesil sülfat poliakrilamid jel elektroforez kullanılarak elde edilen ekstraselülar protein profillerine göre identifiye edilmiştir. Fenotipik özelliklerine göre, yerel izolatların tümünün Gram pozitif, aerobik, endospor oluşturan, hareketli ve fakültatif alkalofilik *Bacillus* cinsine ait türler olduğu belirlenmiştir. Ayrıca, yerel alkalofilik izolatların fenotipik olarak diğer referans *Bacillus* türlerinden farklı olduğu bulunmuştur.

Ekstraselülar protein profil sonuçları esas alınarak yapılan nümerik analiz; yerel izolatlar ve *B. megaterium*'u içeren benzerlik seviyesi % 69 ve % 100 arasında değişen dört grup ve benzerlik seviyesi % 70'in üzerinde diğer referans *Bacillus* türlerini içeren bir grup olmak üzere beş ayrı grup ortaya koymuştur. Bu araştırma, SDS-PAGE yöntemiyle elde edilen ekstraselülar protein profillerinin yerel alkalofilik *Bacillus* izolatları ve diğer *Bacillus* türlerinin taksonomik ilişkilerinin incelenmesinde etkili bir yaklaşım sağladığını göstermiştir.

Anahtar Sözcükler: Van Gölü, identifikasyon, fakültatif alkalofilik *Bacillus* spp., fenotipik karakterizasyon, SDS-PAGE

Introduction

Lake Van is by volume the fourth largest closed body of water on Earth (volume 607 km^3, area 3570 km^2, maximum depth 450 m, lake level 1648 m above sea level, climate, and continental). It is also known as the largest soda lake on Earth with a pH of 9.7-9.8 and a salinity of 21.7‰ contributed to in equal shares by NaCl and sodium carbonates with minor contributions from sulphate, potassium and magnesium (1). It is known that soda lakes are highly alkaline aquatic environments containing a number of alkaliphilic bacteria (2). The genus *Bacillus* consists of alkaliphilic strains, some of which are Gram-positive, endospore-forming, aerobic and facultative anaerobic (3). Recently, studies on the classification and characterization of alkaliphilic *Bacillus* strains have been increasing due to their possessing valuable and commercially interesting enzymes (4). To date, several researchers have studied the identification

and characterization of alkaliphilic *Bacillus* strains based on the phenotypic characteristics, DNA-DNA relatedness data, and phylogenetic analysis of the 16S rRNA sequence (5-9). Although these methods have been used for the classification of alkaliphilic *Bacillus* species, the characterization of these microorganisms is considered complicated (10,11).

Protein electrophoresis is of great value for the delineation of numerous bacterial taxa. Second level information in a cell after the complete nucleotide base sequence of the bacterial genome is given by the cellular proteins, and different types of electrophoresis are used to explore relationships at this level (12). The protein profiles produced by SDS-PAGE of whole cells and extracellular cells of bacteria have been observed to correlate closely with DNA-DNA hybridization results and to be suitable for rapid bacterial identification (13-15).

High resolution polyacrylamide gel electrophoresis (PAGE) of proteins with computerized analysis of profiles provided an effective approach to the investigation of taxonomic relationships among many bacterial species (16,17). In the present study, we aimed to identify 5 reference *Bacillus* species and 17 native alkaliphilic *Bacillus* strains, isolated from the water of Lake Van and the soil surrounding it. They were identified based on phenotypic characteristics and extracellular protein profiles using SDS-PAGE.

Materials and Methods

Reference Bacteria and Growth Conditions

The reference *Bacillus* species (*B. megaterium*, *B. thuringiensis* var. *israelensis*, *B. megaterium* DSM 32, *B. cereus* ATCC 7064 and *B. subtilis*) used in our study was provided by Prof Dr Cumhur Çökmüş (Department of Biology, Faculty of Sciences, Ankara University, Tandoğan 06100 Ankara-Turkey). The reference *Bacillus* strains were grown at 30 °C for 24 h on Nutrient Yeast Salt Medium (Difco) Agar and propagated at least twice before use.

Isolation of Alkaliphilic *Bacillus* spp.

Native alkaliphilic *Bacillus* strains were isolated from the water of Lake Van and the soil surrounding it (Figure 1) according to the isolation procedure described by Horikoshi and Akiba (18). For isolation Alkali Nutrient Agar medium was used. The content of the medium was 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$ $7H_2O$, 2% Agar, pH 10.5. The solution of Na_2CO_3 were autoclaved separately and added to the medium.

Morphological and Phenological Characteristics of the Strains

Phenotypic test studies of the reference strains were used. Native isolated strains in the present study were identified by conventional microbiological methods (18-21). The morphology of vegetative cells and sporangia, and the shape and position of spores were observed under a phase contrast microscope (Nikon, Japan) using ×40 and ×100 magnifications. To keep cells in focus, agar-coated slides were used. In addition, the following phenotypic tests were performed: motility, catalase and oxidase tests; anaerobic growth; Voges-Proskauer test; methyl red test; gas production from glucose; degradation of starch, urea and casein; acid from D-glucose, L-arabinose, D-xylose, D-mannitol, fructose, galactose, maltose, lactose, inulin and sucrose; degradation of tyrosine; deamination of phenylalanine; egg-yolk lecithinase; nitrate reduction to nitrite; formation of indole; H_2S production; DNase test; utilization of citrate; NaCl and KCl required; growth at pH 6.8 and 5.5; growth in 2%, 5%, 7% and 10% NaCl; and growth at 10 ºC, 30 ºC, 40 ºC, 45 ºC and 50 ºC.

Preparation of extracellular proteins

The method given by Wessel and Flügge (22) was used with a few modifications. For each culture, a loopful of overnight growth from an NYSM Agar (for reference *Bacillus* strains) and AN Agar (for native *Bacillus* isolates) plate were suspended in 15 ml of NYSM and AN broth and incubated in a rotated incubator (150 rpm) for 48 h at 30 °C. Samples were then transferred to 1.5 ml Eppendorf tubes and centrifuged for 3 min at 12,000 rpm. After centrifugation, the culture supernatants were transferred to Eppendorf tubes. Afterwards, the culture supernatants were passed through a cellulose acetate membrane filter (Sartorius) with a diameter of 0.25 µm and were stored at -50 °C until the electrophoresis was carried out. A methanol-chloroform precipitation was performed in an Eppendorf tube containing 400 µl of methanol, 200 µl of chloroform and 300 µl of distilled water and the mixture was centrifuged for 3 min at 10,700 rpm. After centrifuging, the upper phase was removed and 300 µl of methanol was added; the mixture

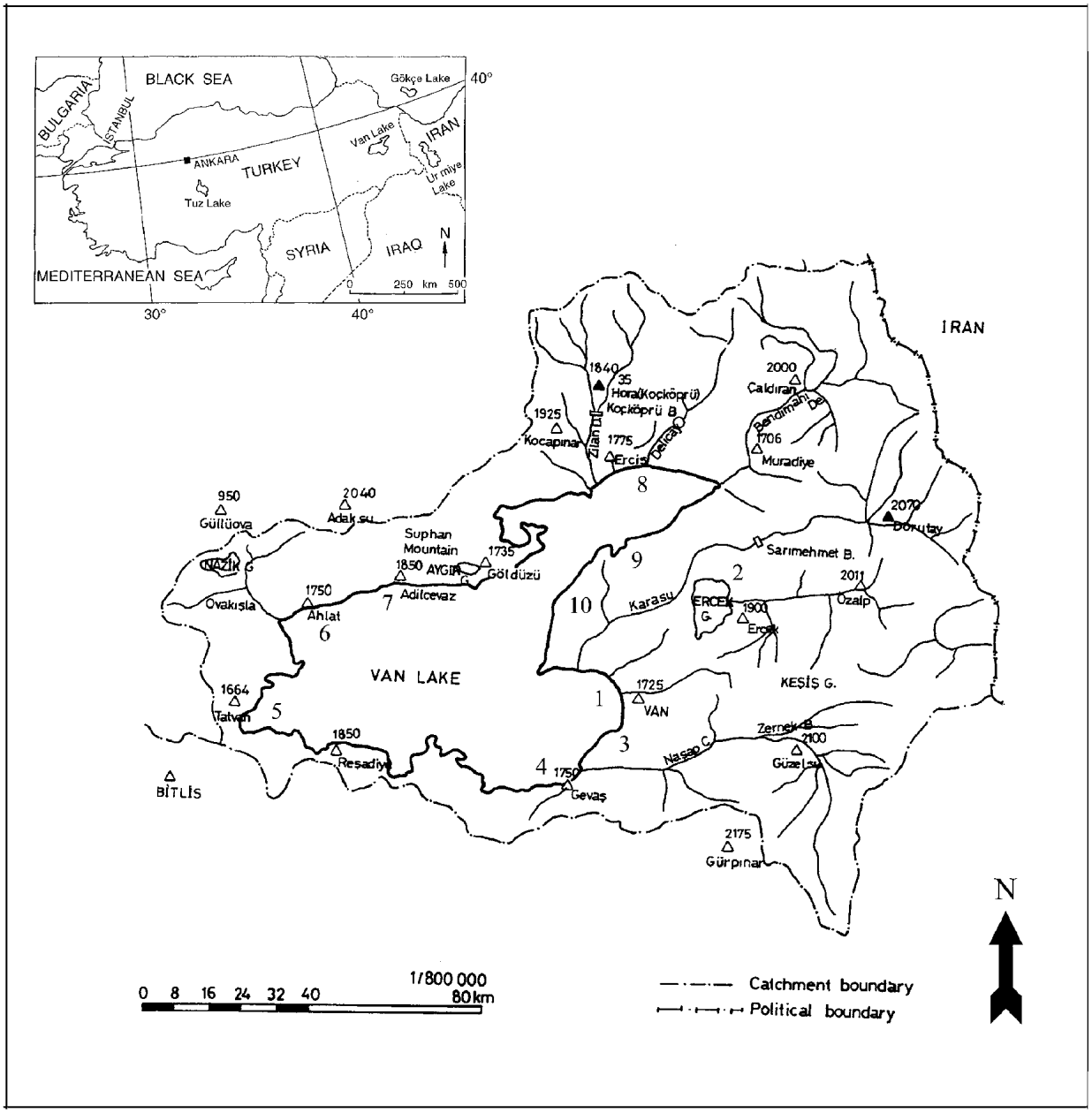

Figure 1. Location of Lake Van, Turkey, and map of the sampling point collected from Lake Van, (1; Campus Area, 2; Lake Erçek, 3; Edremit, 4; Gevaş, 5; Tatvan, 6; Ahlat, 7; Adilcevaz, 8; Erciş, 9; Adır island, 10; Çakırbey).

was stirred and was centrifuged again at 10,700 rpm. Then the supernatant was removed and the precipitated proteins were dried at room temperature. The dried protein was stirred after the addition of 25 µl of SDS-sample buffer (pH 6.8) to the Eppendorf tube. Afterwards, pellet proteins were denatured by keeping them in boiling water for 5 min.

SDS-PAGE

Solubilized proteins were subjected to SDS-PAGE in gel slabs of 1 mm thickness (3.5 cm, 4% stacking and 15.5 cm, 12% resolving gels) as described by Laemmli (23). Electrophoresis was performed with a discontinuous buffer system in a UVP Vertical Electrophoresis Unit Cambridge (UK). The gel was run at

30 mA until the bromophenol blue marker had reached the bottom of the gel. The gels were then stained with Coomassie Brilliant Blue R-250.

Data Analysis

Cluster analysis was performed using STATISTICA software version 6.0 for Windows. This is a program for data input and analysis of binary data and is run on an IBM computer. The similarity and relationship between the protein profiles of the test strains were expressed in terms of a cluster dendrogram based on the unweighted pair group average method with an arithmetic averages algorithm (UPGMA).

Results

According to the morphological and physiological characteristics, all native strains belonged to the genus *Bacillus* genus. The morphological, physiological and biochemical properties of the newly isolated strains are presented in the Table. All strains possessed Gram-positive, aerobic rod, endospore-forming, facultatively alkaliphilic, peritrichously flagellate, motile and catalase positive. Spores were generally ellipsoidal without parasporal crystals and were located central and terminal, swelling in the young sporangium, except one numbered by 36 strain. The strains were cultured in nutrient broth at pH between 6.8 and 10.5 and the optimum pH was 9. This indicated that all strains were facultative alkaliphilic. The ranges of temperature for growth were from 30 to 45 °C. The strains did not grow under 10 °C or above 50 °C. It was observed that all native strains grew in the medium containing 2% and 5% NaCl. However, the strains numbered by 70, 74, 103 and 105 did not grow at 5% NaCl salinity. Other phenotypic characteristics of the strains are in particular the ability to reduce nitrate, use of citrate, production of acid formation from various carbohydrates, hydrolysis of casein, starch and urea, production of H_2S, and gas production from glucose. They also were positive in respect of methyl red and Voges-Proskauer tests, DNase and lecithinase activities listed in the Table.

The extracellular protein profiles of the reference *Bacillus* strains and native facultative alkaliphilic *Bacillus* strains obtained by SDS-PAGE are shown in Figure 2. The protein profiles were inspected visually and compared with each other. The reference *Bacillus* strains yielded profiles that were different from each other and from those of the native facultative alkaliphilic *Bacillus* strains. All of native strains have considerably similar band profiles, particularly in the 20,000-36,000 kDa regions. However, several variations were observed between protein bands of native alkaliphilic *Bacillus* strains. The molecular weight of these bands was above 36,000 kDa. Numbered bands are shown in Figure 2. For instance, isolates 8, 9, 20, 30 and 31 had similar profiles; these isolates were distinguished from the other native isolates and references *Bacillus* in terms of the presence of a single specific band 1 (Figure 2). Furthermore, 2 isolates (4 and 26) had very similar profiles; however, isolate 4 differed from the other isolate by the lack of a major protein band 1 (Figure 2).

The computer-assisted numerical processing of the extracellular protein profiles using cluster analysis with the unweighted pair group method with an arithmetic averages algorithm yielded a dendrogram, which consisted of several groups at similarity levels ranging from 60% to 100% (Figure 3). Based on the electrophoretic mobility of extracellular proteins, the alkaliphilic native strains and reference *Bacillus* species studied were grouped into 5 main clusters (I-V) at the similarity level of 60% as shown in the dendrogram (Figure 3). Group I consisted of 4 reference *Bacillus* strains at the similarity level of 69% (*B. thuringiensis* var. *israelensis*, *B. megaterium* DSM 32, *B. cereus* ATCC 7064 and *B. subtilis*). They were distinguished from the other strains. Group II had 7 tested strains, consisting of only 1 reference *Bacillus* species (*B. megaterium*) and 6 native alkaliphilic *Bacillus* strains (70, 74, 79, 101, 103 and 105). The third group included 2 tested native strains with a similarity level above 75%. Cluster IV included 3 native strains (34, 36 and 42). The similarity levels of the members of this group varied between 84% and 95%. Cluster V consisted of 6 native alkaliphilic strains (4, 8, 9, 26, 30 and 31) with a similarity level of 79-100%.

Discussion

Many studies have suggested that strains of the genus *Bacillus* are more phenotypically heterogeneous than most other bacterial genera (24). There is a diverse group of *Bacillus* species living in highly alkaline terrestrial and aquatic environments. In the past decade there was a full revision of alkaliphilic *Bacillus* classification according to their phylogenetic and

Table 1. Morphological and phenological characteristics of the alkaliphilic *Bacillus* strains isolated from the water of Lake Van and the soil surrounding it.

Characteristics	4	8	9	12	20	26	30	31	34	36	42	70	74	79	101	103	105
Cell shape	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Position of spore	T	T	T	C	T	C	T	T	T	T	T	T	T	T	T	T	T
Sporangium swollen	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+
Parasporal crystals	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flagellation peritrichous	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-
Acid from D-Glucose	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-
" L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" Fructose	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
" Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" Lactose	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
" Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gas from Glucose	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Hydrolysis of Casein	-	-	-	+	-	+	+	+	+	+	-	+	+	+	+	-	-
" Starch	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Degradation of tyrosine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Deamination of Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Egg-yolk lecithinase	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Reduction of nitrate	-	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Formation of Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NaCl and KCl required	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of Urea	-	+	+	-	+	+	+	+	+	+	+	-	-	-	+	-	-
H_2S production	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Growth at pH 6.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 5.5	+	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+	-
DNase	+	+	+	+	-	+	+	+	+	+	+	-	-	+	+	-	-
Utilization of citrate	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Growth in 2% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" 5% NaCl	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-
" 7% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" 10% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 10 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" 30 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" 40 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" 45 °C	+	-	-	-	-	+	-	+	-	-	+	-	-	+	-	-	-
" 50 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Locality no	5	4	4	1	1	4	4	6	3	3	3	9	8	10	2	2	2

Symbols: R: cell form rods; T: terminal spore; C: central spore; +: positive and -: negative

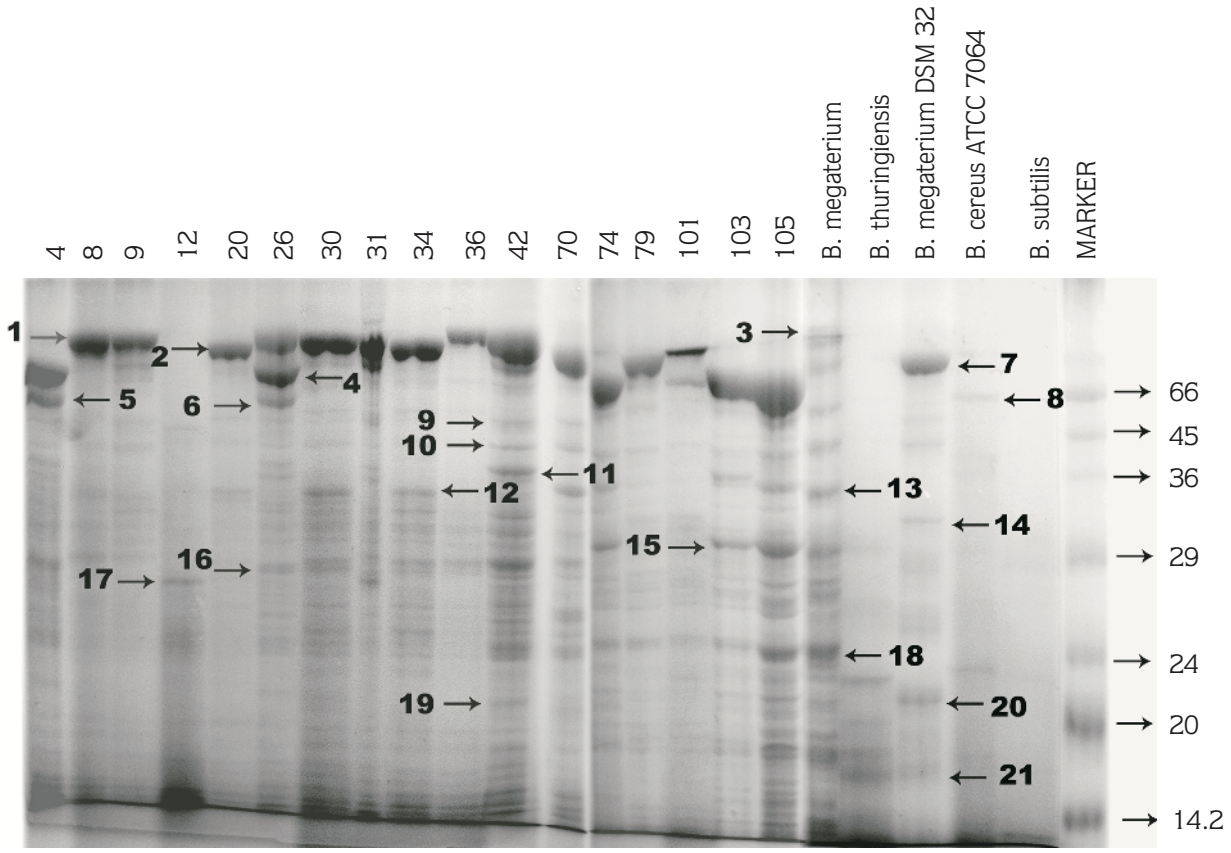

Figure 2. SDS-PAGE of extracellular proteins of 5 reference *Bacillus* species and 17 facultatively alkaliphilic *Bacillus* spp.

phenotypic characteristics (11,20,25). The alkaliphilic and nonalkaliphilic species of the genus *Bacillus* are difficult to identify by traditional methods based on phenotypic characteristics (26).

All the morphological and physiological characteristics of the native strains indicated that these isolates were from in the genus *Bacillus*. Native alkaliphilic *Bacillus* isolates are similar according to the biochemical characteristics. Some native isolates did not utilize much carbohydrate for growth, and no glucose. As a result, it was determined that the conventional tests based on phenotypic characteristics were insufficient for the differentiation of native alkaliphilic *Bacillus* isolates. The results of the numerical analysis of extracellular protein profiles obtained by SDS-PAGE of the native alkaliphilic *Bacillus* isolates and other *Bacillus* strains clearly indicated that the native strains were distinguished from the 4 reference *Bacillus* species. The data presented here led us to the 4 clusters of alkaliphilic *Bacillus* strains, which are similar to *B. megaterium*. Indeed, protein patterns of the native alkaliphilic *Bacillus* strains were closely related to *B. megaterium*. However, other reference *Bacillus* strains formed a cluster that was clearly separated from the other strains. In Addition, the numerical analysis of extracellular protein profiles showed a high similarity between some species, for example, 8 and 9, and 103 and 105. These native isolates also have identical extracellular protein patterns. Their protein profiles indicated, however, that they may represent several new species of this genus.

It is known that the protein profiles of whole-cell and extracellular protein are sufficient for distinguishing at species level for most bacterial genera, but not at the subspecies level (15,27). However, the protein profiles produced by SDS-PAGE of whole cells and extracellular cells of *Bacillus* strains are useful for characterizing these

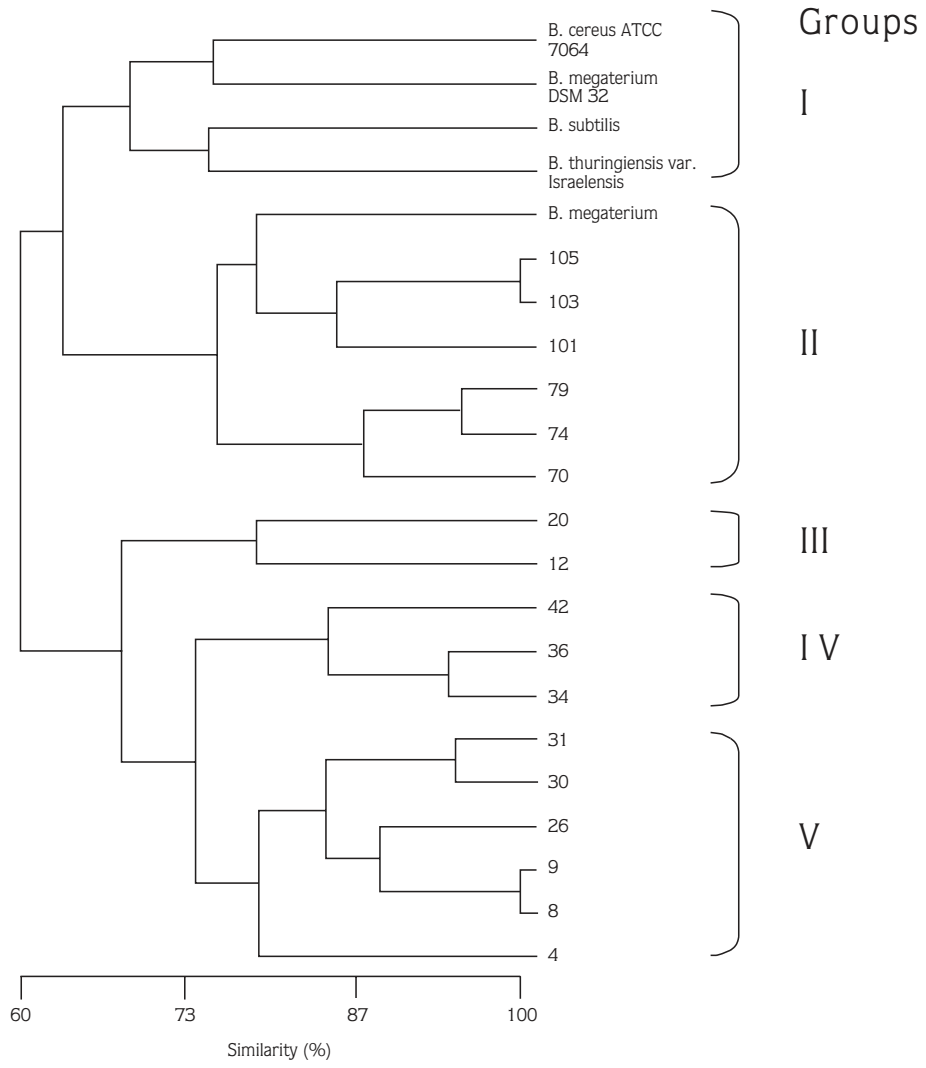

Figure 3. Dendrogram based on the analysis of SDS-PAGE profiles of extracellular of 5 reference *Bacillus* species and 17 facultatively alkaliphilic *Bacillus* spp. by the unweighted pair group average method with an arithmetic averages algorithm (UPGMA).

microorganisms at the species and subspecies level (28-30).

In conclusion, this study showed that the application of numerical analysis, coupled with the utilization of a standardized identification system instead of a simple quantitative comparison of protein patterns, greatly enhances the utilization of extracellular protein profiles for the identification of alkaliphilic *Bacillus* species. Moreover, these results revealed that the numerical analysis of extracellular protein profiles obtained by SDS-PAGE can provide valuable taxonomic information about alkaliphilic and nonalkaliphilic *Bacillus* strains.

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References

1. Kempe S, Kazmlerczak J, Landmann G et al. Largest known Microbialites discovered in Lake Van, Turkey. Macmillan Magazines 349: 605-608, 1991.
2. Granth WD, Jones BE, Mwatha WE. Alkaliphiles: ecology, diversity and applications. FEMS Microbiol Rev 75: 255-270, 1999.
3. Krulwich TA, Guffanti AA. Alkalophilic Bacteria. Ann Rev Microbiol 43: 435-463, 1989.
4. Saeki K, Hitomi J, Okuda M et al. A novel species of alkaliphilic *Bacillus* that produces an oxydatively stable alkaline serine protease. Extremophiles 6: 65-72, 2002.
5. Spanka R, Fritre D. *Bacillus cohnii* sp. nov., a new, obligately alkaliphilic, oval-spore-forming *Bacillus* species with ornithine and aspartic acid instead of diaminopimalic acid in the cell wall. Int Journal Syst Bacteriol 43: 150-156, 1993.
6. Agnew MD, Kowal SF, Jarell KF. Isolation and characterization of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. Syst Appl Microbiol 18: 221-230, 1995.
7. Nielsen P, Fritre D, Priets FG. Phenetic diversity of alkaliphilic *Bacillus* strains. Proposal for nine new species. Microbiology 141: 1745-61, 1995.
8. Fritre D. *Bacillus haloalkaliphilus* sp. nov. International Journal Systematic Bacteriology 46: 98-101, 1996.
9. Yumoto I, Yamazaki K, Hishinuma M et al. Identification of facultatively alkaliphilic *Bacillus* sp. strain YN-2000 and its fatty acid composition and cell-surface aspects depending on culture pH. Extremophiles 4: 285-290, 2000.
10. Ash E, Farrow JAE, Wallbanks S et al. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Letters in Applied Microbiolgy 13: 2002-06, 1991.
11. Muntayan MS, Tourava TP, Lysenko et al. Molecular identification of alkaliphilic and halotolerant strain *Bacillus* sp. FTU as *Bacillus pseudofirmus*. Extremophiles 6: 195-199, 2002.
12. Goodfellow M, O'Donnell A. Handbook of New Bacterial Systematics. Academic Press Limited, London; 1993.
13. Vauterin L, Vantomme R, Pot B et al. Taxonomic analysis of *Xhantomonas campestris* pv. *begonidae* and *X. campestris* pv. *pelargonii* by means of phythopathological, phenotypic, protein electrophoretic and DNA hybridization methods. Systematic and Applied Bacteriol 13: 166-167, 1990.
14. Niemi RM, Niemela SI, Bamford DH et al. Presumptive fecal *Streptococci* in environmental samples characterized by one-dimensional sodium dodecyl sulfate-polyacrilamide gel electrophoresis. App Env Microbiol 59: 2190-96, 1993.
15. Berber I, Cokmus C, Atalan E. Characterization of *Staphylococcus* species by SDS-PAGE of Whole-Cell and Extracellular Proteins. Microbiology 72: 42-47, 2003.
16. Kersters K. Numerical methods in the classification of bacteria by protein electrophoresis. In: Goodfellow M, Jones D, Priest FG et al. Computer Assisted Bacterial Systematic. Academic Press; 1985: pp. 337-368.
17. Costas M, Holmes B, Frith KA et al. Identification and typing of *Proteus penneri* and *Proteus vulgaris* biogroups 2 and 3, from clinical sources, by computerized analysis of electrophoretic protein patterns. J Applied Bacteriology 75: 489-498, 1993.
18. Horikoshi K, Akiba T. Alkalophilic Microorganisms. Japan Scientific Societies Press, Tokyo, Japan, 1982.
19. Gordon RE. One hundered and seven years of the genus *Bacillus*. In: Berkeley RC, Goodfellow M. ed. The aerobic endosporeforming bacteria. Academic Press, London; 1981:pp.322-344.
20. Fritze D, Flossdorf J, Clause D. Taxonomy of alkaliphilic strains. International Journal Systematic Bacteriology 40: 92-97, 1990.
21. Slepecky RA, Hemphill HE. The Genus *Bacillus* Nonmedical. In: Balows A, Trüper HG, Drowkin, et al. ed. The Prokaryotes. Springer-Verlag, London; 1992: pp.1662-1695.
22. Wessel D, Flugge UI. A method for the quantitative recovery of protein in dilute solution presence of detergents and lipids. Anal Biochem 138: 141-143, 1984.
23. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680-85, 1970.
24. Claus D, Berkeley CW. The Genus *Bacillus*. In: Sneath PHA ed. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore; 1986: pp.1105-1139.
25. Takami H, Han C, Takaki et al. Identification and distribution of new insertion sequences in the genome of alkaliphilic *Bacillus halodurans* C-125. Journal of Bacteriology 183: 4345-56, 2001.
26. Woese CR. Bacterial Evolution. Microbiological Reviews 51: 221-271, 1987.
27. Maggs AF, Pennington TH. Temporal study of *Staphylococcal* species on skin of human subjected in isolation and clonal analysis of *Staphylococcus capitis* by sodium dodecyl sulphate polyacrylamide gel electrophoresis. J Clin Microbiol 27: 2627-32, 1988.
28. Cokmus C, Yousten AA. Characterization of *Bacillus sphaericus* strains by SDS-PAGE. J Invertebr Pathol 64: 267-268, 1987.
29. Berber I, Cokmus C. Characterization of *Bacillus sphaericus* strains by Native-PAGE. Bull Pure Appl Sci 20: 17-21, 2001.
30. Berber I. Characterization of *Bacillus* species by numerical analysis of their SDS-PAGE protein profiles. J Cell Mol Biol 3: 33-37, 2004.