NUMERICAL ANALYSIS OF SDS-PAGE PROTEIN PATTERNS OF FACULTATIVE ALKALIPHILIC *BACILLUS* SPECIES ISOLATED FROM LAKE VAN, TURKEY

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SUMMARY

In the present study, seven reference *Bacillus* species and a total of eighteen new facultative alkaliphilic *Bacillus* strains, isolated from the water of Lake Van and its soil surroundings, were identified using phenotypic characteristics and the numerical analysis of whole-cell protein profiles. According to morphological, biochemical and physiological characteristics, it was found that the new isolated strains belonged to *Bacillus* genus. In addition, the research indicated that SDS-PAGE of polypeptides of whole-cell extracts can provide more valuable taxonomic information than conventional tests based on the phenotypic characteristics at the species and subspecies levels. Numerical analysis of whole-cell protein profiles of test strains revealed 4 basic clusters (I-IV) at dissimilarity values of 18.9 % or above. The results of numerical analysis confirmed that each cluster had characteristic and distinctive protein profiles. Indeed, this study showed that the application of numerical analysis, coupled with the utilization of a standardized identification system instead of simple quantitative comparison of protein patterns, greatly enhanced the utilization of whole-cell protein profiles for identification of the facultative alkaliphilic *Bacillus* species.

KEYWORDS: Lake Van, *Bacillus* spp., numerical analysis, protein profiles, phenotypic characterization, SDS-PAGE.

INTRODUCTION

Lake Van, the world's largest soda lake (volume 607 km^3, area 3570 km^2, maximum depth 450 m, lake level 1648 m above sea level, continental climate) is located on the Anatolian high plateaus in eastern Turkey (38.5ºN and 43ºE). It has a pH of 9.7-9.8, and a salinity of 21.7‰ contributed to in equal shares by NaCl and sodium carbonates, and with minor contributions from sulfate, potassium and magnesium [1]. Soda lakes are highly alkaline aquatic environments containing a number of alkaliphilic bacteria [2]. Up to now, many of the microorganisms characterized from soda lakes have relatives in salt lakes, except those being alkaliphilic or, at least, highly alkali-tolerant [3]. It was reported that *Bacillus* genera consist of alkaliphilic strains, some of which are Gram-positive, endospore- forming, aerobic and facultative anaerobic [4]. In the last decade, the taxonomical studies on alkaliphilic *Bacillus* strains are rising due to possessing valuable and commercially interesting enzymes [5].

The conventional identification and classification methods based on morphological, physiological and biochemical properties can clearly lead to misclassification in some bacterial taxa. Due to this fact, the development and use of new molecular methods for improving the identification and detection of microorganisms are advisable [6, 7].

Some researchers have studied identification and characterization of alkaliphilic *Bacillus* strains based on the phenotypic characteristics, DNA-DNA relatedness data, and phylogenetic analysis of the 16S rRNA sequence [8-11]. Although these methods have been used for classification of alkaliphilic *Bacillus* species, the characterization of these microorganisms is still considered to be complicated [6, 12].

Protein electrophoresis has been of great value for the delineation of numerous bacterial taxa. The primary level of information for identification of bacteria is given by nucleotide base sequencing. Second level information is given by the cellular proteins, and different types of electrophoresis are used to explore relationship at this level [13]. SDS electrophoresis in a discontinuous system is, by far, the most widely used electrophoretic technique in bacterial systematics. The protein profiles produced by SDS-PAGE of whole cells and extra-cellularly by bacteria have been observed to correlate closely with DNA-DNA hybridization results, and to be suitable for rapid bacterial identification [14-17].

Computer-aided numerical analysis of protein patterns has a high potential in microbial systematics. 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 [18, 19]. The aim of the present study is to identify seven reference *Bacillus* species and eighteen new alkaliphilic *Bacillus* strains, isolated from the water of Lake Van and the soil of its surroundings. The strains were identified, based on phenotypic characteristics and the numerical analysis of whole-cell protein profiles.

MATERIALS AND METHODS

Bacteria and growth conditions

The reference bacteria used in this study were provided from various researchers: *B. megaterium* GCM 1842, *B. megaterium* DSM 32 and *B. cereus* ATCC 7064 from Prof. Dr. Cumhur Cokmus (Department of Biology, Faculty of Sciences, Ankara University, Tandoğan 06100 Ankara, Turkey), *B. sphaericus* MRS 400 from Prof. Dr. Allan A. Yousten (VPI & State University, Blacksburg, VA, USA), and *B. firmus* ATCC 14573, *B. pseudofirmus* OF4 and *B. alcalophilus* ATCC 27647 from Dr. Arthur A. Guffantı and Dr. Terry A. Krulwich (Department of Biochemistry, Mont Sinai School of Medicine of the City University of New York, 10029 New York, USA). The nonalkaliphilic strains were cultivated at 30 °C and pH 7.5 for 24 hrs in Nutrient Yeast Salt Broth (NYSM) medium consisting of 0.8% nutrient broth (Difco), 0.05% yeast extract (Difco), $CaCl_2$ x $2H_2O$ - 7.0 x 10^{-4} M, $MnCl_2$ x 4 H_2O - 5.0 x 10^{-5} M, and $MgCl_2$ x 6 H_2O - 1.0 x 10^{-3} M, whereas the alkaliphilic strains were propagated at 30 °C and pH 7.5 for 24 hrs in Peptone Yeast Alkali Broth (PYA) medium consisting of 1% soluble starch (Difco), 0.5% polypeptone (Difco), 0.5% yeast extract (Difco), 0.1% K_2HPO_4, 0.02% $MgSO_4 \times 7H_2O$, and 0.5% Na_2CO_3. The solution of Na_2CO_3 were autoclaved separately and added to the medium.

Isolation of alkaliphilic *Bacillus* spp.

New facultative alkaliphilic *Bacillus* strains were isolated from the water of Lake Van and the soil of its surroundings (Figure 1) by using the isolation procedure of Horikoshi and Akiba [20], and PYA Agar medium (1% soluble starch (Difco), 0.5% polypeptone (Difco), 0.5% yeast extract (Difco), 0.1% K_2HPO_4, 0.02% $MgSO_4 \times 7H_2O$, 0.5 % Na_2CO_3, 2% Agar, pH 10.5).

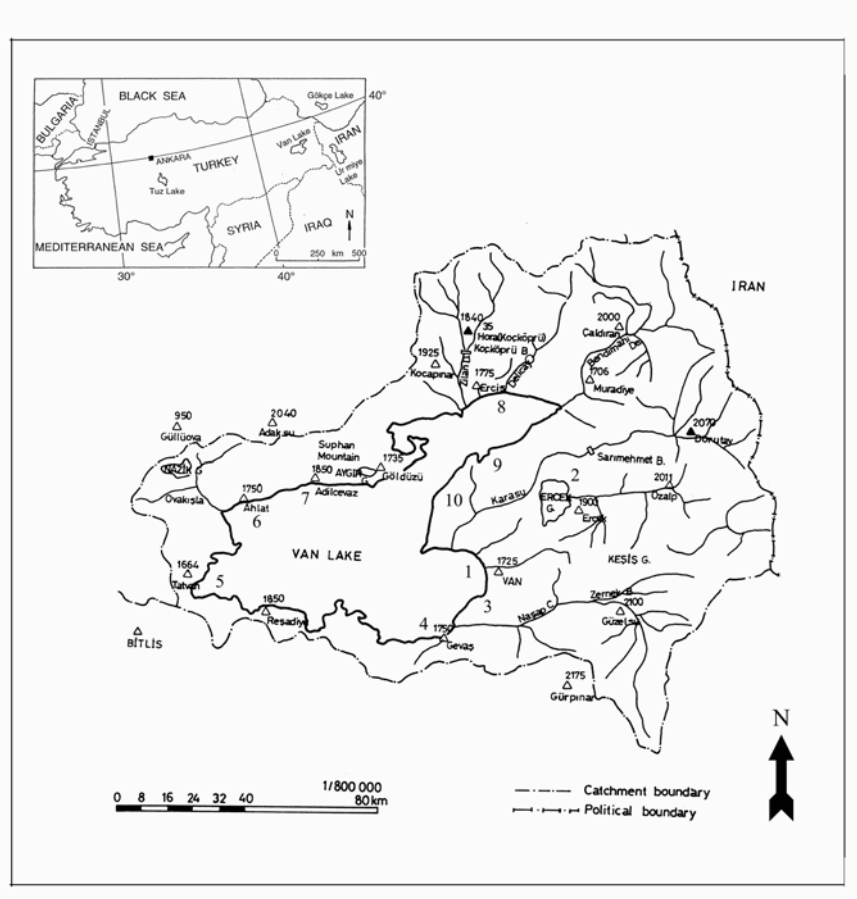

FIGURE 1 - Location of Lake Van, Turkey and map of the sampling points.
(1 Campus Area, 2 Lake Ercek, 3 Edremit, 4 Gevas, 5 Tatvan, 6 Ahlat, 7 Adilcevaz, 8 Ercis, 9 Adır island, 10 Çakırbey).

Physiological and morphological properties of the strains

For the phenotypic test studies, the following reference strains were used: *B. megaterium* GCM 1842, *B. megaterium* DSM 32, *B. sphaericus* MRS 400, *B. cereus* ATCC 7064, *B. firmus* ATCC 14573, *B. pseudofirmus* OF4, and *B. alcalophilus* ATCC 27647. New isolated strains in the present study were identified by conventional microbiological methods [20-22]. The morphology of vegetative cells and sporangia, and the shape and position of spores were observed under a phase contrast microscope (Nikon, Japan). In addition, the following phenotypic tests were performed: motility, catalase and oxidase test; 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 reduced to nitrite; formation of indole; H_2S production; DNase test; utilization of citrate; NaCl and KCl required; growth at pHs 6.8 and 5.5 of nutrient broth; growth in NaCl 2%, 5%, 7% and 10%; and growth at 10, 30, 40, 45 and 50 ºC.

Antibiotic sensitivity

Sensitivity to antibiotics was determined by using the routine diffusion plate technique. Reference *Bacillus* strains (Nutrient Yeast Salt Agar medium at pH 7.5) and alkaliphilic strains (Peptone Yeast Alkali Broth medium at pH 7.5) were grown overnight at 30 °C, and used to prepare suspensions with optical density of 0.5 McFarland Standard (1.5 x 10^8 cells per mL). 100 µL of each test bacterium was plated onto the agar, and disks containing antibiotics were placed onto the surface of the medium. After overnight-incubation at 30°C, the diameters of the zones of growth inhibition were measured. The following antibiotics were used (µg/disk): imipenem (10 µg), ceftizoximine (30 µg), ciprofloxacine (5 µg), cefodizime (30 µg), ofloxacine (5 µg), amoxicillin and clavulanic acid (10 µg), and sulbactam/ ampicillin (20 µg).

Extraction of whole-cell proteins from bacteria

All test strains were, at least, propagated in duplicate to prepare the synchronous culture. For each synchronous culture, 100 µL was inoculated into 15 mL NYSM or PYA broth, and incubated under rotation for 48 hrs (at 30 °C, 150 rpm). Each sample was centrifuged for 5 min at 12, 100 rpm, and the pellet collected was resuspended in 200 µL of CelLytic™ B-II Bacterial Cell Lysis/Extraction Reagent (Sigma) [23]. The suspension was incubated for 30 min at room temperature. Afterwards, the sample was again centrifuged and 80 µL from each sample was transferred into a new 1.5 mL Eppendorf tube. Then, 25 µL of SDS-samples` buffer (0.06 M Tris-HCl, 2.5% glycerol, 0.5% SDS, 1.25% β-mercaptoethanol) was added and the whole mixture was vortexed to ensure good homogenization. The prepared samples were kept on a boiling water bath for 5 min and denaturated proteins stored at -70 °C until electrophoresis.

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 [24]. Electrophoresis was performed with a discontinuous buffer system in an 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. Protein molecular masses were calculated on the basis by comparison with the following standards (Prestained SDS-PAGE Standards, High Range, BIO-RAD): myosin (201 kDa), β-galactosidase (118 kDa), bovine serum albumin (83 kDa), and ovalbumin (48 kDa). After electrophoresis, the gels were rinsed out for 20 min in an isopropanol-acetic acid-water (1:3:6) solution, then for 5 min in methanol-acetic acid-water (3:1:6) solution. Then, the gels were stained for 6 hrs in 0.01 % (w/v) Coomassie Brilliant Blue R-250. Afterwards, the gels were destained in a methanol-acetic acid-water (3:1:6) mixture, until protein bands became clearly visible.

Statistical analysis of protein profiles

The gels were scanned via densitometer (Desaga CD-60 Densitometer, Germany), and the molecular weight of each band was determined by one-dimensional analysis software (Lab Image Version 2.6, Halle, Germany). Data were coded as 0 (absent) and 1 (present). A hierarchical cluster analysis was performed using the average linkage method with the squared Euclidean distances [25]. The dendrogram, based on the whole-cell protein patterns of the test strains, was constructed by the program SPSS for Windows, version 12.0 (Chicago, Ill., USA).

RESULTS AND DISCUSSION

In the present research, eighteen facultative alkaliphilic *Bacillus* strains isolated from the water of Lake Van and its surrounding soil were examined. Morphological and physiological characteristics revealed that the new strains were all members of *Bacillus* genus. Morphological, physiological and biochemical properties of newly isolated strains are presented in Table 1. The data show that all strains are Gram-positive, aerobic rod, endospore-forming, facultative alkaliphilic, peritrichously flagellate, motility and catalase-positive. Spores were generally ellipsoidal without parasporal crystals, and located central and terminal, swelling in the young sporangium, except two strains numbered by 57 and 107. The strains were cultivated in nutrient broth at pHs between 6.8 and 10.5, and optimum pH was found to be 9. This case indicated that all strains were facultative alkaliphilic. All the new strains were able to grow at temperature from 20 °C to 40 °C. The optimum growth temperature was 30 °C, but no growth was observed under 10 °C and above 50 °C. The native strains showed salt tolerance at NaCl concentrations upto 5%. But, the strains numbered by 70, 74, 103 and 105, did not grow at 5% NaCl salinity. Other phenotypic characteristics of the strains were

determined to have the particular ability to reduce nitrate, utilization of citrate, acid formation from various carbohydrates, hydrolysis of casein, starch and urea, H_2S production, gas production from glucose, methyl red and Voges-Proskauer tests, and DNase and lecithinase activities (listed in Table 1). The results of antibiotic tests indicated

TABLE 1 - Morphological and phenotypical properties of the native and reference *Bacillus* strains.

Properties	42	79	101	70	74	77	86	103	105	107	4	8	9	30	31	34	40	57	*B. pseudofirmus* OF4	*B. firmus* ATCC 14573	*B. alcalophilus* ATCC 27647	*B. sphaericus* MRS 400	*B. megaterium* DSM 32	*B. cereus* ATCC 7064	*B. megaterium* GCM 1842
SDS-PAGE lane numbers[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Cell shape	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Position of spore	T	T	T	T	T	T	T	T	T	C	T	T	T	T	T	T	T	C	T	C	T	T	C	C	C
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	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
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″ Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
″ Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-
Gas from Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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″ 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 10ºC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
″ 30ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
″ 40ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
″ 45ºC	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-
″ 50ºC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Antibiotics susceptibility[b]:																									
Imipenem	27	30	29	33	35	30	36	32	33	35	29	30	30	33	27	35	26	25	48	46	35	20	36	42	44
Ceftizoximine	24	28	22	30	30	32	33	27	18	22	30	21	21	27	23	26	21	20	40	9	26	27	14	31	40
Ciprofloxacine	20	30	29	25	25	26	25	25	26	30	24	24	23	22	19	27	15	14	38	35	32	15	26	40	34
Cefodizime	13	18	16	21	21	24	23	21	9	22	25	17	17	18	13	19	11	10	28	17	20	33	11	26	33
Ofloxacine	16	26	24	24	27	20	25	23	23	24	20	20	20	19	18	23	15	16	30	30	26	12	24	32	33
Amoxicillin-and clavulanic acid	20	24	23	28	28	32	30	25	27	22	26	27	27	22	22	25	22	21	40	40	25	12	12	28	26
Sulbactam-ampicillin	8	9	8	17	8	-	10	9	-	11	8	10	10	10	8	8	7	8	15	15	9	10	8	19	19
Locality no	3	10	2	9	8	8	1	2	2	5	4	4	4	6	3	3	3	7							

R: cell form rods; T: terminal spore; C: central spore; +: positive and -: negative; [a]Numbers correspond to the lane in Fig. 2; [b]Diameter of inhibition zone (mm)

that reference *Bacillus* strains and native isolates had almost equal sensitivity against the tested antibiotics. Nevertheless, native strains were fairly resistant against sulbactam-ampicillin antibiotic.

The analysis of whole-cell protein profiles of the reference and new facultative alkaliphilic *Bacillus* strains, obtained by one-dimensional denaturing gel electrophoresis, are shown in Figure 2. The protein profiles of tested bacterial strains were inspected visually and compared with each others. The protein profiles of the test strains revealed considerable differences; however, there were some similar protein bands between new strains and reference *Bacillus* strains. The test strains have curiously distinctive protein bands in the range of molecular weights between 83-201kDa.

The numerical analysis of the whole-cell protein profiles used for average linkage method with the squared Euclidean distances yielded a dendrogram, consisting of four basic clusters (I-IV) at dissimilarity values of 18.9 % or above (Figure 3). Cluster I comprised *B. sphaericus*

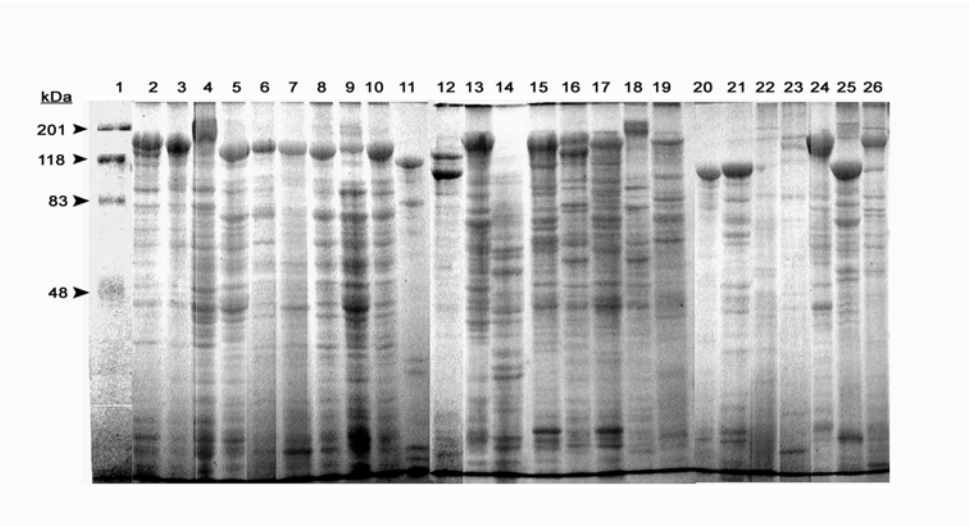

FIGURE 2 - SDS-PAGE of whole-cell protein profiles of the reference and the native Bacillus strains. Lane1, molecular weight standards (kDa); lanes 2-19, the native strains; lines 20-26, the references strains. Lanes are identified in Table 1[a].

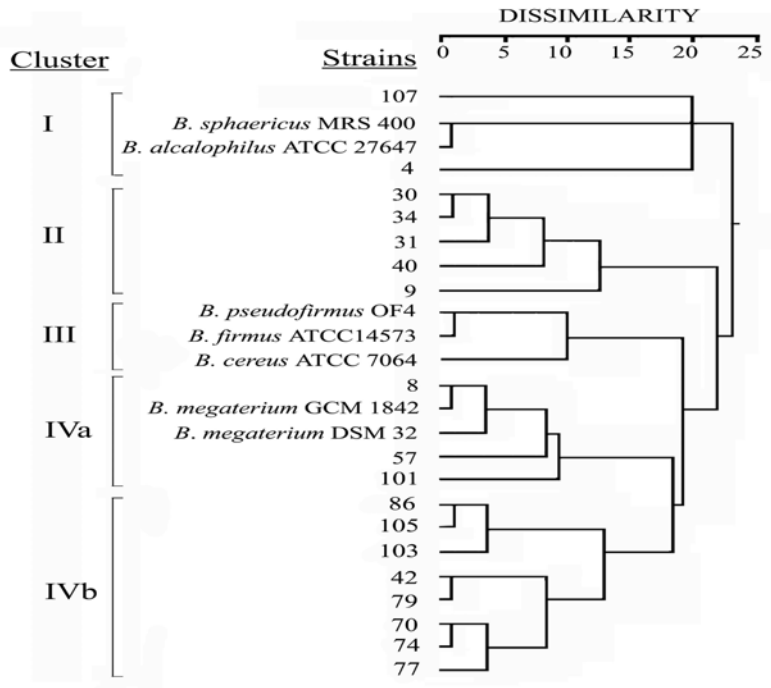

FIGURE 3 - Grouping of the reference and the native Bacillus strains studied using cluster analysis (the squared Euclidean distances and average linkage clustering method) based on whole-cell protein profiles.

MRS 400, *B. alcalophilus* ATCC 27647 and two new facultative alkaliphilic strains, numbered as 4 and 107, at dissimilarity level between 0.8 % and 20 %. Cluster II had 5 facultative alkaliphilic strains numbered as 9, 30, 31, 34 and 40. The dissimilarity levels of the members of this group changed between 0.9 % and 12.4 %. The third cluster included three reference strains (*B. pseudofirmus* OF4, *B. firmus* ATCC 14573, and *B. cereus* ATCC 7064), which had 10 % of maximum distance. Cluster IV was divided in two subclusters (IVa and IVb) including *B. megaterium* GCM 1842, *B. megaterium* DSM 32 and 11 new strains (8, 42, 57, 70, 74, 77, 79, 86, 101, 103 and 105). Those strains were similar to *B. megaterium* GCM 1842 or *B. megaterium* DSM 32. Besides, the members of this cluster had the highest similarity of protein profiles.

A number of researchers have reported that the strains of the genus *Bacillus* are recognized as being more phenotypically heterogeneous than most other bacterial genera [26]. Truly, this genus exhibits a wide diversity of physiological ability, since the members of the genus are able to live in very different kinds of habitats. There is a diverse group of *Bacillus* species living in highly-alkaline terrestrial and aquatic environments. In the past decade, a full revision of alkaliphilic *Bacillus* classification was marked according to their phylogenetic and phenotypic characteristics [12, 21, 27]. The species of alkaliphilic and nonalkaliphilic *Bacillus* genera are notoriously difficult to be identified by traditional methods, based on their physiological and morphological properties [6, 7, 12].

According to the present study, morphological, biochemical and physiological characteristics indicated that the new isolated strains are closely related to *Bacillus* genus. The strains had almost similar biochemical characteristics. For instance, they did not utilize much carbohydrate for growth, except for glucose. Although the new isolated strains have been extensively investigated as a representative strain of facultative *Bacillus*, their taxonomic position is not yet known. As a result, it was determined that the conventional tests based on the phenotypic characteristics were insufficient for the differentiation of the native isolates.

Great attention has been given to protein electrophoresis, for the identification of a number of bacterial genera [14]. It is also widely acknowledged that the electrophoretic separation of cellular proteins is a sensitive technique, which mainly provides information on the similarity of the strains at and below the species level. The results of this study obviously showed that the electrophoretic method can provide more valuable information than phenotypic methods at and below the species levels, and may be used for the discrimination of some new isolated and other *Bacillus* strains. The results are in good agreement with previous studies, which observed that some *Bacillus* species displayed distinct band variation in their SDS-PAGE whole-cell protein patterns at the species and subspecies level [28, 29].

Analysis of whole-cell protein profiles of the *Bacillus* strains is useful for characterizing these microorganisms at the species and below the species level. However, computer-aided numerical analysis of protein patterns has provided more useful information at subspecies level [14, 17]. In the present study, SPSS program was used to analyze the data because of the difficulties in the visual interpretation of the bands, obtained in SDS-PAGE of whole-cell proteins. According to the results of the numerical analysis of whole-cell protein extracts, the new *Bacillus* isolates scattered distinctly within the reference *Bacillus* species. The results of numerical analysis confirmed that each cluster had characteristical and distinctive protein profiles. For example, the members of clusters IVa and IVb had the highest similarity of protein profiles related to *B. megaterium* DSM 32 strain (Fig. 2). The members of Cluster II had very similar protein profiles. However, there were some minor band variations. Besides, protein profiles from particular strains of the *B. alcalophilus* ATCC 27647 and *B. sphaericus* MRS 400 species were put into a distinct cluster (Fig. 3). Cluster III included three reference strains (*B. pseudofirmus* OF4, *B. firmus* ATCC 14573, and *B. cereus* ATCC 7064), and it was slightly differed from Clusters IVa and IVb. The data presented here led the author to make four main clusters of new alkaliphilic *Bacillus* strains, which are similar to different reference *Bacillus* strains. Indeed, numerical analysis of one-dimensional SDS-PAGE of protein profiles of the native and reference strains was concluded to provide more useful approach towards clarifying relationship within *Bacillus* species, compared to visual inspection of protein bands.

In conclusion, this study showed that the application of numerical analysis, coupled with the utilization of a standardized identification system, instead of simple quantitative comparison of protein patterns, greatly enhanced the utilization of whole-cell protein profiles for identification of the native and reference *Bacillus* species. In addition, the protein patterns of the isolated facultative alkaliphilic *Bacillus* strains possibly represent new species of this genus. The numerical analysis of whole-cell protein profiles obtained by SDS-PAGE can provide valuable taxonomic position about new facultative alkaliphilic *Bacillus* strains.

ACKNOWLEDGMENTS

This research was supported by Scientific and Technical Research Council of Turkey (TBAG-2103 (101T146). We would like to thank Prof Dr. Cumhur Cokmus, Dr. Arthur A. Guffantı, Dr. Terry A. Krulwich and Prof. Dr. Allan A. Yousten for reference *Bacillus* strains.

REFERENCES

[1] Kempe, S., Kazmierczak, J., Landmann, G., Konuk, T., Reimer, A. and Lipp, A. (1991). Largest known Microbialites discovered in Lake Van Turkey. Macmillan Magazines 349: 605-608.

[2] Grant, W.D., Jones, B.E. and Mwatha, W.E. (1990). Alkaliphiles: ecology, diversity and applications. FEMS Microbiol. Rev. 75:255-270.

[3] Jones, B.E., Granth, W.D., Collins, N.C. and Mwatha, W.E. (1994). Alkaliphiles: diversity and identification. In: Priest, F.G., Ramos-Carmenzana, A. and Tindall, B.J. (eds) Bacterial Diversity and Syst. Plenum Press, New York, 195-229.

[4] Krulwich, T. and Guffanti, A.A. (1989). Alkalophilic Bacteria. Ann. Rev. Microbiol. 43:435-463.

[5] Saeki, K., Hitomi, J., Okuda, M., Hataka, J., Kageyama, Y., Takaiwa, M., Kubota, H., Hagihara, H., Kobayashi, T., Kawai, S. and Ito, S. (2002). A novel species of alkaliphilic *Bacillus* that produces an oxydatively stable alkaline serine protease. Extremophiles 6: 65-72.

[6] Ash, C., Farrow, J.A.E., Wallbanks, S. and Collins, M.D. (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Lett. Appl. Microbiol. 13:2002-2006.

[7] Ivanova, P.E., Vysotskii, M.V., Svetashev, V.I., Nedashkovskaya, O.I., Gorskova, N.M., Mikhailov, V.V., Yumoto, N., Shigeri, Y., Taguchi, T. and Yoshikawa, S. (1999). Characterization of *Bacillus* strains of marine origin. Int. Microbiol. 2:267-271.

[8] Spanka, R. and Fritre, D. (1993). *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. J Syst. Bacteriol. 43:150-156.

[9] Nielsen, P., Fritre, D. and Priets, F.G. (1995). Phenetic diversity of alkaliphilic *Bacillus* strains. Proposal for nine new species. Microbiology 141:1745-1761.

[10] Fritre, D. (1996). *Bacillus haloalkaliphilus* sp. nov. Int. J. Syst. Bacteriol. 46.98-101.

[11] Yumoto, I., Yamazaki, K., Hishinuma, M., Nodasaka, Y., Inoue, N. and Kawasaki, K. (2000). 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.

[12] Muntayan, M.S., Tourava, T.P., Lysenko, A.M., Kolganova, T.V., Fritze, D. and Skulachev, V. (2002). Molecular identification of alkaliphilic and halotolerant strain *Bacillus* sp. FTU as *Bacillus pseudofirmus*. Extremophiles 6:195-199.

[13] Vauterin, L., Swings, J. and Kersters, K. (1993). Protein Electrophoresis and Classification. In: Goodfellow, M. and O' Donnell, A.G. (Eds.) A Handbook of New Bacterial Systematics, Academic Press Limited, London, pp.251-272.

[14] Vauterin, L., Vantomme, R., Pot, B., Hoste, B., Swings, J. and Kersters, K. (1990). Taxonomic analysis of *Xhantomonas campestris* pv. *begonidae* and *X. campestris* pv. *pelargonii* by means of phythopathological, phenotypic, protein electrophoretic and DNA hybridization methods. Syst and Appl. Bacteriol. 13:166-167.

[15] Nıemi, R.M., Nıemela, S.I., Bamford, D.H., Hantula, J., Hyvarınen, T., Forsten, T. and Raateland, A. (1993). Presumtive Fecal *Streptococci* in Environmental Samples characterized by One- Dimensional Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis. App. and Env. Microbiol. 59: 2190-2196.

[16] Berber, I., Cokmus, C. and Atalan, E. (2003). Characterization of *Staphylococcus* species by SDS-PAGE of Whole-Cell and Extracellular Proteins. Microbiology 72:42-47.

[17] Berber, I. (2004). Characterization of *Bacillus* species by Numerical Analysis of their SDS-PAGE Protein Profiles. J. Cell and Mol. Biol. 3:33-37.

[18] Costas, M., Holmes, B., Frith, K.A., Riddle, C. and Hawkey, P.M. (1993). Identification and typing of *Proteus penneri* and *Proteus vulgaris* biogroups 2 and 3, from clinical sources, by computerized analysis of electrophoretic protein patterns. J. Appl. Bacteriol. 75:489-498.

[19] Zarnowski, R., Eichel, J., Lewicka, T., Rozyckı, H. and Pietr, S.J. (2001). Protein fingerprinting as a complementary tool for the classification of *Pseudomonas* bacteria. Cell & Mol. Biol. Lett. 6:913-923.

[20] Horikoshi, K. and Akiba, T. (1982). Alkalophilic Microbiologorganisms. Japan Scientific Societies Press, Tokyo, Japan.

[21] Fritze, D., Flossdorf, J. and Clause, D. (1990). Taxonomy of alkaliphilic strains. Int. J. Syst. Bacteriol. 40:92-97.

[22] Slepecky, R.A. and Hemphill, H.E. (1992). The Genus *Bacillus* Nonmedical. In: Balows, A., Trüper, H.G., Dworkin, M. Harder, W. and Schleifer, K.H. (Eds.) The Prokaryotes. Springer-Verlag, London pp.1662-1695.

[23] Mehigh, R. (2001). Lysis of *E. coli* prufication of soluble recombinant proteins using CelLytic-A™ and CelLytic-B™ II. Sigma Origins 3:15-16.

[24] Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

[25] Ten Bosch, J.J., Wan der Mei, H.C. and Busscher, H.J. (1991). Statistical analysis of bacterial species based on physicochemical surface properties. Biofouling 4:141-150.

[26] Claus, D. and Berkeley, C.W. (1986). The Genus *Bacillus*. In: Sneath, P.H.A. (Ed.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore pp.1105-1139.

[27] Takami, H., Han, C., Takakı, Y. and Ohtsubo, E. (2001). Identification and Distribution of New Insertion Sequences in the Genome of Alkaliphilic *Bacillus halodurans* C-125. J. Bacteriol. 183:4345-4356.

[28] Zheng, G. and Slavik, M.F. (1999). Isolation, partial purification and characterization of a bacteriocin produced by a newly isolated *Bacillus subtilis* strain. Lett. Appl. Microbiol. 28: 363-367.

[29] Berber, I. and Cokmus, C. (2001). Characterization of *Bacillus sphaericus* strains by Native-PAGE. Bull. Pure and Appl Sci. 20:17-21.

Received: September 12, 2005
Accepted: November 07, 2005

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